Biologic Structure and Function: Perspectives on Morphologic Approaches to the Study of the Granular Layer Keratinocyte

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Stephen Rothman [1] wrote: ''Modern dermatology has been built on the solid pillars of precise macro- and microscopic observation, accurate recording and meaningful interpretation of the findings. No natural science can exist without direct observation of natural phenomena, and dermatology has become a great discipline because we have had so many good observers.'' He went on to state further that ''… whereas the immense knowledge acquired by the classical descriptive methods is still rapidly increasing, the application of experimental methods to dermatologic problems is a relatively young and undeveloped approach.'' These statements acknowledge the importance of observation and description (usually thought of as features of morphology) in clinical dermatology and in descriptive dermatologic research, but were made before he could recognize the value of morphology to experimental studies as well. Unfortunately, Dr. Rothman did not see the remarkable expansion in dermatologic research that has taken place within even the last 20 years, an era in which descriptive biology and experimental biology are not necessarily separate entities or even parallel concepts. Morphology is simply one of many approaches—sets of tools—that can be used to solve problems in biology.

Morphology is often the starting point of an investigation. Understanding the structure of a cell or tissue provides a framework on which other kinds of data can be applied. A morphologic study, however, need not be synonomous with description and qualitative investigation. Many of the techniques used by morphologists provide compositional information (presence/absence of a chemical compound) on a structural substrate (location within the cell or tissue) which, in turn, permits interpretation of function. For example, electron microscopic autoradiography can be used to identify the site of incorporation of a radiolabeled amino acid withm the tissue and the specific cell and organelle in which it is utilized, or x-ray microanalysis can reveal the elemental composition of parts of cells or different cells within a tissue. Through the use of techniques like morphometry and stereology, morphologic studies can also be quantitative. To continue the example from above, the volume density of the radiolabeled organelle can be determined for a cell or tissue by means of selective sampling and prescribed methods of counting, thus allowing for quantitation and statistical analysis when comparing experimental data. Morphology should not have the limited connotation of providing purely descriptive information.

The structure and composition of a tissue can provide insight into its function. As stated by Braverman and Braverman [2], ''a major aim of biological work is to correlate structure with function in order to understand how tissues work.'' The goal in the present paper, as indicated by its title, is to support this statement as we review the use of morphologic approaches to understand skin biology, selecting as an illustration the progress that has been made in understanding the structure, composition and function of one cell, the granular layer keratinocyte. This approach avoids describing the advances in methodology, and, instead, demonstrates how morphology has promoted the conceptual development of the topic (Tables I–III).

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WHAT DO MORPHOLOGIC METHODS OFFER? AN OVERVIEW

The development of morphologic approaches for skin research has involved the development of instrumentation (to a large extent, microscopy) methods to prepare tissue for morphologic examination (e.g., biopsy, separated epidermis and dermis, isolated cells, cell fractions, extracted samples, cultured or grafted specimens, etc.) and techniques to preserve (e.g., chemically fixed) and process (e.g., embedded, freeze-dried) specimens in as natural a state as possible, free of distortion and artifact. Development of methodolgoy for tissue preparation is as important as the development of microscopes with high resolving power; the use of sophisticated instruments to examine skin specimens is irrelevant if the tissue is poorly preserved and thus provides only limited information. This can be appreciated by noting the lack of clarity and detail in the electron micrographs of the investigators in the 1950s and comparing them with the relatively high resolution images of cell structure obtained by investigators in even the late 1960s. The latter were prepared after reasonably good techniques of fixation and embedding were available. Once the basic problems of specimen processing for microscopy were resolved, new, specialized methods were developed that at allowed for the identification of specific components (histochemistry, cytochemistry, enzymatic extraction, immunolabeling, x-ray microanalysis, etc.), the demonstration of different cellular compartments (e.g., intramembranous planes by freeze-fracture), and assessment of viability (supravital stains) at the tissue (histologic) and cellular (electron microscopic) levels.

To review both the instrumentation and morphologic methods in the context of skin research would be equivalent to reviewing progress in the methods developed and used by morphologists over the last 50 years, an unwieldy topic of enormous dimension and little interest. However, a cursory review is important for an investigator who may wish to understand the range of approaches that can provide morphologic information. Table IV has been prepared as an overview which includes examples that demonstrate how a method has been used to provide new information about skin structure, composition and/or function.

A MORPHOLOGIC PROFILE OF THE GRANULAR LAYER KERATINOCYTE PROVIDES: STRUCTURAL, COMPOSITION AND FUNCTIONAL INFORMATION

The granular layer is defined primarily on a structural basis. It is recognized by its position as the uppermost two—three layers of the viable epidermal compartment and by darkly stained or electron dense

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Table I. Evolution of Understanding of the Keratohyalin Granule Based on Morphologic Evidence

Concepts of Keratohyalin Granule Structure and Function	Morphologic Evidence
KHGs are large, dark staining granules present in the upper epidermal layers	Light microscopic observations of sections of epidermis
KHGs may be degradation products of keratinization	Similarities in staining with other structures in the tissue, based on histologic preparations
Mammalian KHGs granules have a particulate substructure, rodent KHGs are heterogeneous in structure	Electron microscopic observations of KHGs in fixed, embedded samples of epidermis
KHGs are associated with filaments	Electron microscopic observation of embedded tissue; extraction of the dense material in the KHG with acid, alkali or buffer reveals filaments which are then identified by immunocytochemical staining with anti-keratin antibodies
Mammalian KHGs are not sulfur-rich	Histochemical staining for SH groups and S-S bonds in tissue; sections and isolated KHGs; ^{3}H-cystine fails to localize specifically in the granular layer when examined by autoradiography
A small KHG in rodent epidermis is sulfur-rich	High sulfur peak occurs over ''single granules'' when tissue samples are analyzed by energy dispersive x-ray microanalysis; ''single granules'' are positive when reacted with silver methenamine and examined by electron microscopy
KHGs are histidine-rich	Strong staining of the granular layer and KHGs with the histochemical stain for histidine (Pauly reaction); specific uptake of ^{3}H-histidine by cells of the granular layer
KHGs bind metallic ions	Selective binding of radiolabeled Zn; KHGs are strongly reactive with solutions of other cations as demonstrated by staining reactions of tissue sections
KHGs are sites of active protein	An association with ribosomes is noted by electron microscopy; selective uptake synthesis of ^{3}H-histidine and other labeled amino acids occurs in labeled animals and isolated tissue sections; mRNA for profilaggrin is localized in the granular layer by *in situ* hybridization
The protein of the KHG is related to a protein in the stratum corneum	Translocation of ^{3}H-histidine from the KHG to the stratum corneum in animals injected with label or tissues incubated in label; cross-reactivity of protein in the KHG and stratum corneum when the tissue is stained immunohistochemically with antibodies to stratum corneum matrix protein or KHG protein; similarity in reaction of the KHGs and lower cornified cells with staphylococcal protein A in glutaraldehyde-fixed tissue
KHG-derived protein can ''glue'' and keratin filaments	Similar fine structure is noted when comparing KHG in align transitional cells and lower stratum corneum cells; interaction between stratum corneum matrix protein and keratin filaments *in vitro* leads to the formation of aligned macrofibrils that have a ''keratin pattern''-like organization when examined by electron microscopy

granules now known to be the keratohyalin granules (Fig 1). Currently we have a clear view of the structure—and we understand a great deal about the function—of the granular cell and its intermediate position between a metabolically active and a dying cell. This was not always the case. This level of understanding was attained gradually as new information about each of the cellular components was unravelled, through morphologic investigation, and separate or coordinated studies of epidermal and granular layer biochemistry. Descriptive morphology at the light and electron microscopic levels has set the stage for further investigation into each of the elements imaged: keratohyalin granules, lamellar granules, filaments, plasma membrane and the nucleus. Each of these has been studied in the intact tissue or isolated for study of the structure apart from its relationship with other cellular components. The evolution in our understanding the structure, composition and function of these structures will be reviewed as a means of demonstrating the role morphology has played in advancing our understanding of skin biology. Admittedly, by focusing primarily on morphologic data in our discussion of the granular cell, a substantial amount of information will be missing. This is particularly notable when considering the molecular composition of the keratohyalin granule and keratin filaments, but the boundaries of this discussion are set by the charge to review the development of morphologic approaches in dermatologic research rather than to comprehensively review the various aspects of the granular layer keratinocyte.

Keratohyalin Granules (Table I) The most conspicuous structure within the granular cell is the keratohyalin granule (KHG), named by Waldeyer in 1882 and described by light microscopists as ''round specks, coarse irregular lumps, drops or rods''[3]. Some thought they were formed in the granular cell at the expense of mitochondria; others suggested they originated from the Golgi, the ground substance, or from tonofibrils by ''fibrillorhexis,'' as reviewed in [4]. Similarities in staining of KHGs and chromatin with basic dyes led to the concept that KHGs may be breakdown products of the nucleus [5]. However, stains specific for DNA (Feulgen) revealed that DNA was not a component of the KHG [6]. RNA was considered an alternative to account for the basophilia, but, except for the observed (by transmission electron microscopy) association of ribosomes with the periphery of the KHG (particularly notable in the rodent KHG), there was no evidence from histochemical staining or other methods of analysis to suggest that RNA was a significant component [7]. Nonetheless, the apparent relationship of RNA with the KHG helped to focus the perceived function of the keratohyalin granule in the right direction: KHGs may be active sites of synthesis rather than the byproducts of degradation [6]. This was not a new concept. In 1879 Ranvier described the granular layer as a ''keratogenic layer'' [4]. How the keratohyalin granule is involved in keratinization has been unravelled through studies that have increased our understanding of its structure, chemical composition and metabolism.

The ultrastructure of the KHG supported the concept that it is related to ''keratin.''[1] The smaller KHGs of the deeper granular layer cells were observed to ''grow'' [8] in size progressively toward the stratum corneum [9] (Fig 1), forming large masses of electron dense substance within the

[1]The early definition of ''keratin'' equated keratin with the content of the stratum corneum cell.

Table II. Evolution of Understanding of the Lamellar Granule Based on Morphologic Evidence

Concepts of Lamellar Granule Structure and Function	Morphologic Evidence
Lamellar granules are membrane bound organelles, with a lamellated internal structure, found in the upper epidermal layers	Electron microscopic observation of fixed and embedded samples of epidermis and oral epithelium
Laternal contents appear to be flattened discs or liposomes	Electron microscopic observations of freeze-fracture replicas of the lamellar granule in epidermis
Lamellar granules are lipid-rich	Assumed correlation of the structure as seen by electron microscopy; confirmed by fluorescence microscopy using a fluorochrome that recognizes lipids in general; specific classes of lipids identified by histochemical staining of tissue sections and isolated cell fractions containing lamellar granules; reaction of tissue with digitonin reveals the presence of free sterols when tissue is processed for freeze fracture and replicas are observed by electron microscopy
Lamellar granules contain hydrolytic enzymes	Histochemical reactions demonstrate the presence of specific hydrolytic enzymes
Calcium ions are stored in lamellar granules	Ion capture cytochemistry using oxalate-antimonate-osmium precipiate technique reveals electron dense deposits in the lamellar granules when seen by electron microscopy
The lamellar granule is the source of the lipids that form the intercellular lamellae at the granular-stratum corneum interface and within the stratum corneum	Electron microscopic observation shows that lamellar granules become associated with the plasma membrane and release their contents into the extracellular space by exocytosis; there is a similarity in organization of the lamellae within the lamellar granule and the initial disk-like organization of material in the intercellular space as seen by electron microscopy in routine and freeze-fractured specimens; histochemical staining of lipids in the granular and cornifed cell layers indicates the relationship of lipids in the two layers
The lipids of the lamellar granule are remodeled in the intercellular space	Histochemical staining of lipids and enzymes indicates that the appropriate enzymes are present to alter the polar lipids of the lamellar granule to the neutral lipids of the intercellular space
The content of lamellar granules does **not** coat the membrane	Electron microscopic observation failed to confirm a relationship between the material expelled from the lamellar granule and that which reinforces the plasma membrane in a submembranous location
The lamellar granule derived lipids of the intercellular space for the epidermal permeability barrier	Tracers injected into the skin and allowed to percolate upward or placed on the skin and allowed to penetrate downward are blocked at the level of the intercellular lipid lamellae

Table III. Evolution of Understanding of the Keratin Filament Based on Morphologic Evidence

Concepts of Keratin Filament Structure and Function	Morphologic Evidence
Anisotropic fibrils are present in the viable epidermal cells	Light microscopic observation
Filaments in epidermal cells are artifacts	Light microscopic observations of living cells; low resolution electron microscopic observation of fixed and embedded epidermal cells
Filaments are present in epidermal cells of all layers; individual filaments and loose bundles of filaments occur in basal cells, filaments are consolidated in suprabasal cells and are even more densely packed in cornified cells; filaments are oriented according to the major axis of the cell	Light and electron microscopic observations of epidermal cells in fixed, embedded samples of skin
Filaments in all layers have similar molecular structure	X-ray diffraction of epidermal protein from viable epidermal layers and the stratum corneum
Keratin filaments consist of protofibrils and protofilaments	High resolution electron microscopic observation of negatively stained and shadowed preparations of reconstituted and variably unravelled keratin filaments
Filaments serve a cytoskeletal role providing both mechanical strength and flexibility; they provide a counterbalance to distorsion, assist in centration of the nucleus and implement cell-cell contact	Light and electron microscopic observation of filaments in epidermis and cultures keratinocytes; immunofluorescent staining of keratin filaments in cultured keratinocytes revealing their architectural organization
Filaments play a role in keratinization	Light and electron microscopic observations of keratins in all epidermal layers; immunohistochemical labeling of keratins in all epidermal layers demonstrating their continuity at all stages of differentiation; relationship with KHGs demonstrated by enzymatic digestion of keratohyalin and immunostaining of unmasked filaments remaining within the KHG remnants
Different classes of keratin filaments are expressed in cells according to the type of epithelium and its embryonic origin, state of differentiation, extrinsic conditions, body site, disease state and species	Immunohistochemical labeling with anti-keratin monoclonal antibodies recognize different classes of keratins in different tissue sections and cultured cells

transitional cell, a cell that is intermediate between the granular and the cornified cell [10]. This suggested a gradual and sequential evolution of keratohyalin as it became incorporated into the keratin of the cornified cell Keratohyalin granules were also observed to be associated with filaments (tonofibrils) [8,11–14] in a manner that was reminiscent of the association between filaments and the dense substance in stratum

Table IV. Summary of Morphologic Methods as Applied to Studies of Skin Biology

Special Instruments, Techniques for Morphologic Research	Examples of Use of Technique in Skin Investigation
Microscopy	
Routine light microscopy	
Brightfield	To examine histologic preparations
Darkfield	To emphasize silver grains revealing location of radiolabeled molecules; cDNA probes [41]
Fluorescence microscopy	To excite fluorochromes used as general [126] or specific stains (e.g., lipids-ANS, [75]), as labels for probes such as antibodies or lectins e.g., [123]; or to recognize an ion (e.g., calcium with chlorotetracyclin [81]; two fluorochromes may be used to label a tissue simultaneously; stereopairs of fluorescent images may be obtained to reveal three-dimensional appearing relationships [109]; staining can be enhanced and fading prevented by including para-phenylenediamine in the mounting buffer [127]
Nomarski differential interference optics	To provide visual depth to surface structure; used with frozen sections to enhance cell boundaries and spatial organization (cell stacking) inherent in swollen, alkali-treated epidermis [128]
Polarizing microscopy	To evaluate birefringent properties characteristic of oriented, isotropic structures; demonstrated lattice-like structure of tonofibrils in the epidermis of callus [100]; changes in tonofibril density, orientation from basal to granular layer in accord with the changing cell shape in normal epidermis; to reveal deviations in normal fibril orientation in dyskeratotic and malignant lesions [129]
Scanning laser acoustic microscopy (SLAM)	To visualize boundaries in and properties of skin based on differences in ability of the tissue to propagate ultrasonic waves; information reflects material properties of the tissue such as connective tissue, water content and order; has been used to study wound healing and skin pathology [130]
Transmission electron microscopy (TEM)	To evaluate at high resolution the morphology of cells, organelles, extracted components, tissue fractions and molecules prepared by a variety of techniques; TEM negatives also have been used to quantitatively analyze the density of a structure using a densitometer, e.g., desmosome structure [131] or used in optical diffractometry to obtain information about the periodicity of a structure (e.g., the nonvesicular portion of the Langerhans cell granule [132]); can be used in combination with image analysis devices
Scanning electron microscopy (SEM)	To visualize three-dimensional, topographic structure of cells, tissues, extracted components, cell particles etc. at high resolution; can be used in combination with image analysis devices
Scanning transmission electron microscopy (STEM)	To obtain quantitative, compositional information in the context of its position within the tissue [133–135]; primary method used with x-ray microprobe and energy dispersive x-ray microanalysis; also used to obtain data on the mass of a structure using linear density measurements of the imaged structure, e.g., mass of intermediate filaments [97]
X-ray Diffraction	To reveal information about the molecular structure of crystalline objects thereby to assist in understanding molecular interactions; has been used to demonstrate periodic patterns of extracted, stretched and unstretched keratin [20,92–94]; reconstituted keratin filaments [95], keratin from patients with harlequin ichthyosis [136], stretched crystalline lipid in stratum corneum cells [137], and to show that the lipid patterns characteristic for the stratum corneum reflect the total composition of the lipids in the intercellular space [138]
Physical Changes in Tissue Composition and Organization Prior to Morphologic Examination	
Epidermal-dermal separation	The epidermis and dermis can be separated at the dermal-epidermal junction by a variety of methods (heat, enzymes, etc.); controlled separation of the epidermis below the granular layer can be accomplished by the injection of staphylococcal epidermolytic toxin [64]; the epidermal sheets and dermis then can be used independently in experiments and studied by various morphologic techniques
Epidermal cell separation	Cells can be separated enzymatically and sorted with a cell sorter [139], or by panning, rosetting (e.g., for Langerhans cells), density gradient or velocity sedimentation, or by sequential trypsinization. Isolated preparations are easily pelleted and embedded and sectioned or affixed to slides for microscopic examination. Tape stripping is also commonly used to remove stratum corneum cells, layer by layer; has been used to compare sizes of cells in normal [140] vs. pathologic tissue (e.g., uninvolved and involved areas of psoriasis patients; [141])
Cell fractionation	Fractions of cells are examined morphologically to confirm the presence of a specific organelle (e.g., lamellar granule [72–74]) and/or labeled compound incorporated by the tissue prior to separation and fractionation [27]
Biochemical isolation; reconstitution	Chemically isolated components can be examined as crude or purified preparations (e.g., keratohyalin [27,40,50]; keratin [142]), after reconstitution into a structural entity (e.g., keratin filaments, [95]) or after reaction *in vitro* of two or more components (e.g., keratin filaments and filaggrin [61] or a protein (keratin) and divalent cations [143]
Replica formation	Replicas of the skin surface, or of faces of frozen, fractured tissue, can be prepared for light and electron microscopic examination of topographic morphology. This can be accomplished noninvasively by the application of cyanoacrylate to the skin surface. Replicas of stratum corneum cells (for example) were prepared at progressively deeper layers and examined by SEM in order to correlate anatomic change with properties of cell cohesion [144]; (see also freeze fracture)
Swelling	Treatment of tissue with alkaline solutions [145] causes swelling of the stratum corneum allowing visualization of tissue architecture [128,146] and ease in cell counting [147]
Freeze fracture	Frozen tissue is fractured in vacuuo and a replica of the fractured or etched (controlled sublimation of ice) surface is prepared by carbon and metal shadowing. Fracture planes are frequently within a membrane revealing detail about the internal surfaces of the membrane leaflets. The technique has been used to compare structure of plasma membranes and intercellular junctions of different epidermal layers [85,86], normal versus diseased epidermis (e.g., psoriasis, [148]) and lamellar granules [69]; pre-treatment of the tissue with markers for specific lipids of membranes (e.g., filipin treatment to mark cholesterol sites in membranes) can reveal differences in the chemical nature and distribution of molecules within various cell membranes [149]

Table IV. Continued

Special Instruments, Techniques for Morphologic Research	Examples of Use of Technique in Skin Investigation
Analysis of Tissue Composition Chemical Compounds	
Histochemistry/cytochemistry/ bioassay	There are many tissue and molecule-specific stains which have played an important part in understanding the composition of a region of the skin and the processes that are carried out therein. These reactions can be carried out in tissues (histochemistry) and/ or at the level of the cell (cytochemistry); some of the reactions are suitable for visualization at both levels (150) Histochemical reactions used prominently in the skin are those which demonstrate the position of sulfur containing proteins by recognizing sulfhydryl groups and disulfide bonded molecules (e.g., Barnnett and Seligman method, [24]; nitroprusside method; [21,22]; the thiol reagent DACM, [151]), the presence of histidine (Pauly reaction [30]), lipids [75] and carbohydrate moieties. Several components can also be visualized at the EM level, e.g., sulfur containing compounds (silver methenamine reaction [57]); carbohydrate moieties (e.g., PAS stain [152] and PA-TCH [77]); lectins (120–122), enzymes (76); lipids (75,80); and DNA (Schiff thallium [115])
Immunohistochemistry/ immunocytochemistry	Used in the same manner as histochemistry to localize specific compounds in tissue or cells; has relied upon the development of antibodies as probes for identification of specific tissue components, structures or markers. Where antibodies are available, immunohisto(cyto)chemical procedures have largely replaced histochemistry because of their greater accuracy, variation in marker substances (fluorochromes, ferritin, colloidal gold), precision of localization (small, precise tags such as colloidal gold can be used), application at both the light and electron microscopic levels, hence high resolution of information; can be used in combination with other techniques.
Autoradiography	Used to identify the site of a radioactively labeled molecule that has been administered to an organism *in vivo* or to cells or tissue *in vitro*. The metabolism and possible relocation of that molecule in the tissue can be followed over time. The use of ^{3}H-thymidine has been particularly powerful in identifying mitotic cells and their progeny, determining cell cycle and cell transit times, and revealing epidermal stem cells [153]; it is also useful for localizing the site and following the course of metabolism of a specific organic compound incorporated *in vivo* (e.g., labeled ^{3}H-histidine and ^{3}H-cytidine incorporation into keratohyalin granules of the granular cells [36]). Many kinds of radiolabeled probes can be used (see below for additional examples)
Molecular biology/*in situ* hybridization	Used to identify the site of transcription of a specific gene using a cDNA probe; can be paired with immunocytochemical studies of the same or a parallel tissue sample to determine where in the tissue the message and product are expressed—whether both are expressed in the same cell of if there is differential regulation of the synthesis and expression of message. A radiolabeled probe is used hence autoradiography is a component of the procedure; has been used to show that the expression of profilaggrin mRNA and synthesis of profilaggrin protein occur coordinately in the granular cell layer [41]
Extraction/digestion	To recognize, indirectly, the chemical composition of structures and organelles in the cell; can be done by digesting pre-embedded tissue or using oxidized thin sections of post-embedded specimens with enzymes (pronase, trypsin, pepsin, RNAse, DNAse, phospholipase C [17], and subtilisin [154], buffer (e.g., keratohyalin, [40]) or alkali then examined by electron microscopy; can also unmask structures not seen routinely (e.g., filaments within keratohyalin granules [15,16])
Ion localization	
Microininceration	A forerunner technique to nondestructive elemental microanalysis, whereby fixed or unfixed tissue is heated to very high temperatures leaving a ''skeletal residue'' of the tissue; the organic components are removed but the topographic relationships in the tissue are preserved and the inorganic components (except for S and P) are retained. The mineral content is identified by the color of the residue and/or reaction of the residue with other compounds or dyes [155]; the method has been used to compare mineral content of the different epidermis layers and to compare epidermis from normal and diseased tissue [156]
Ion capture cytochemistry	A method used to localize calcium stores in the tissue, at the cellular level, that depends upon the precipitation and formation of an insoluble residue of calcium with oxalate and pyroantimonate. Sites of calcium ion are seen ultrastructurally as electron dense deposits (81)
X-ray microprobe analysis/ Energy dispersive x-ray analysis	Sensitive methods to analyze, qualitatively or quantatively, individually or simultaneously, the fixed and diffusible elements present in tissues, cells and structures within cells; analysis is carried out by collecting characteristic x-rays emitted from specimens bombarded by an electron beam. Sections, extracted material and/or whole mount specimens are imaged in a TEM or STEM during the analysis; the method has been used to compare elements and diffusible ions in different epidermal layers in normal [157,158] and diseased epidermis [134,159], to assay for the presence of S in various components of the granular cell [56], to evaluate the role of Ca and S in the hardness of the nail [133], to compare elemental preservation using various modes of tissue fixation [160], and to evaluate the water concentration across the epidermis [161]
Proton (particle)-induced X-ray emission PIXE	A highly sensitive method for simultaneous analysis of elements with an atomic number greater than 14 using freeze dried sections of skin bombarded with protons instead of electrons; because of the limited impedence of protons in passing through the specimen (hence negligible background) the method is highly sensitive for heavy elements and trace elements which can be measured to a level of 1 ppm. A profile of elements across the normal and psoriatic epidermis has been made using PIXE [162] and measurement of the trace elements in hair [135] have obtained using this method
Other methods to determine tissue mass (see also STEM)	X-ray images of paraffin-embedded sections can be prepared using historadiography. The amount of x-ray absorption, seen as white on the film, is proportional to the mass which can be measured by a densitometric trace. This methods has been used to compare density across the epidermis [163]
Analysis of Functional Properties	Autoradiography has been mentioned as one morphologic method to trace the metabolism of a specific molecule or compound administered through the cell and tissue with time. Another method to follow dynamic events in cells includes the use of tracer compounds to follow pathways of permeability across the epidermis/through the skin. Lanthanum nitrate, ferritin and thorium dioxide have been used as tracers to follow the intercellular space when incubated with tissue slides or injected intracutaneously *in vivo* and allowed to permeate into the tissue. Use of this method demonstrated the epidermal barrier to coincide with the lamellar granule-derived intercellular lipid lamellae [86]

Table IV. Continued

Special Instruments, Techniques for Morphologic Research	Examples of Use of Technique in Skin Investigation
Tissue and Cell Quantitation	
Measurement/morphometry/ image analysis	Using careful sampling techniques and statistical analysis, studies are done to quantify the density of a structure. This can be performed at the light microscopic (histometry) or EM level. These methods have been used to demonstrate, for example, the density of attachment structures at the dermal-epidermal junction [164]; the size and overlap of populations labeled by ^{3}H-thymidine and anti-69 kDa keratin with position among epidermal strata in normal and psoriatic epidermis [165], the comparative volumes of nuclei among cells of the viable epidermal layers [166]; epidermal cell size and surface area in normal [167] and pathologic epidermis [141]; changes in size of various epidermal compartments after time in organ culture [168], and to compare epidermal cell size versus density [169]; some of the studies used computer-assisted measurement and analysis (e.g., [167])
Stereology	A method which uses morphometric data from EM and LM two dimensional images to calculate the volumetric distribution of cellular components. These studies provide objective, quantitative data that may be used to compare the cytoarchitecture of different layers in normal [170] versus pathologic tissue and to demonstrate the volume density of one organelle [64,74]
3-D reconstruction	Three dimensional structures (e.g., vasculature, cells, or parts of cells such as the Langerhans cells granule [171,132]) may be reconstructed using information in serially sectioned tissue photographed at the light or EM level. Profiles of the structure of interest are traced and the tracing are either entered into a computer (computer-assisted reconstruction), or used to make models from string [172], balsa, wood or wax. The method has been particularly useful to demonstrate in three-dimension, the histology and ultrastructure of normal cutaneous vasculature (e.g., [173]), vascular lesions (e.g., [172]), and the vessel wall [2]
Use of Morphologic Data in Model Building	Several types of morphologic data (sometimes in combination with biochemical data) have served as the basis for building working models of various cutaneous structures. This has been done, for example, to prepare models of intermediate [96,97] and keratin filament molecular structure [98], structure of all or portions of the Langerhans cell granule [132,171], membrane organization in lamellar granules and intercellular lipid sheets [69] and to explain the precursor product relationships between the lipids of the lamellar granules and those in the intercellular space [75,80]

Figure 1. Transmission electron micrograph showing keratohyalin granules three layers of granular cells (1–3). Note their increasing size toward the epidermal surface and their association with filaments. ×17,500

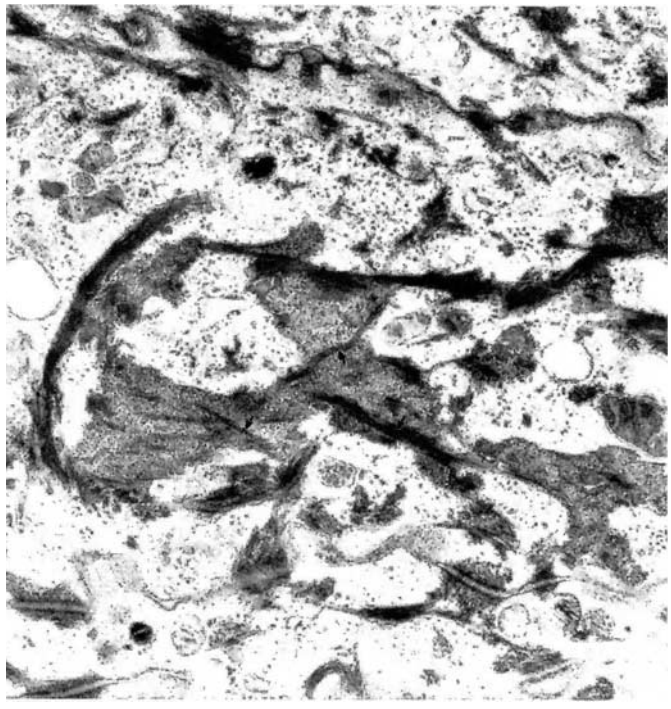

Figure 2. Transmission electron micrograph showing filaments (arrows) within the substances of several keratohyalin granules. ×33,750

corneum cells. Charles and colleagues [12,13] reported that the substance of keratohyalin is deposited on tonofibrils which remain visible within this material as ''lighter lines'' embedded within a dense matrix. This description foreshadowed accurately the ''keratin pattern''

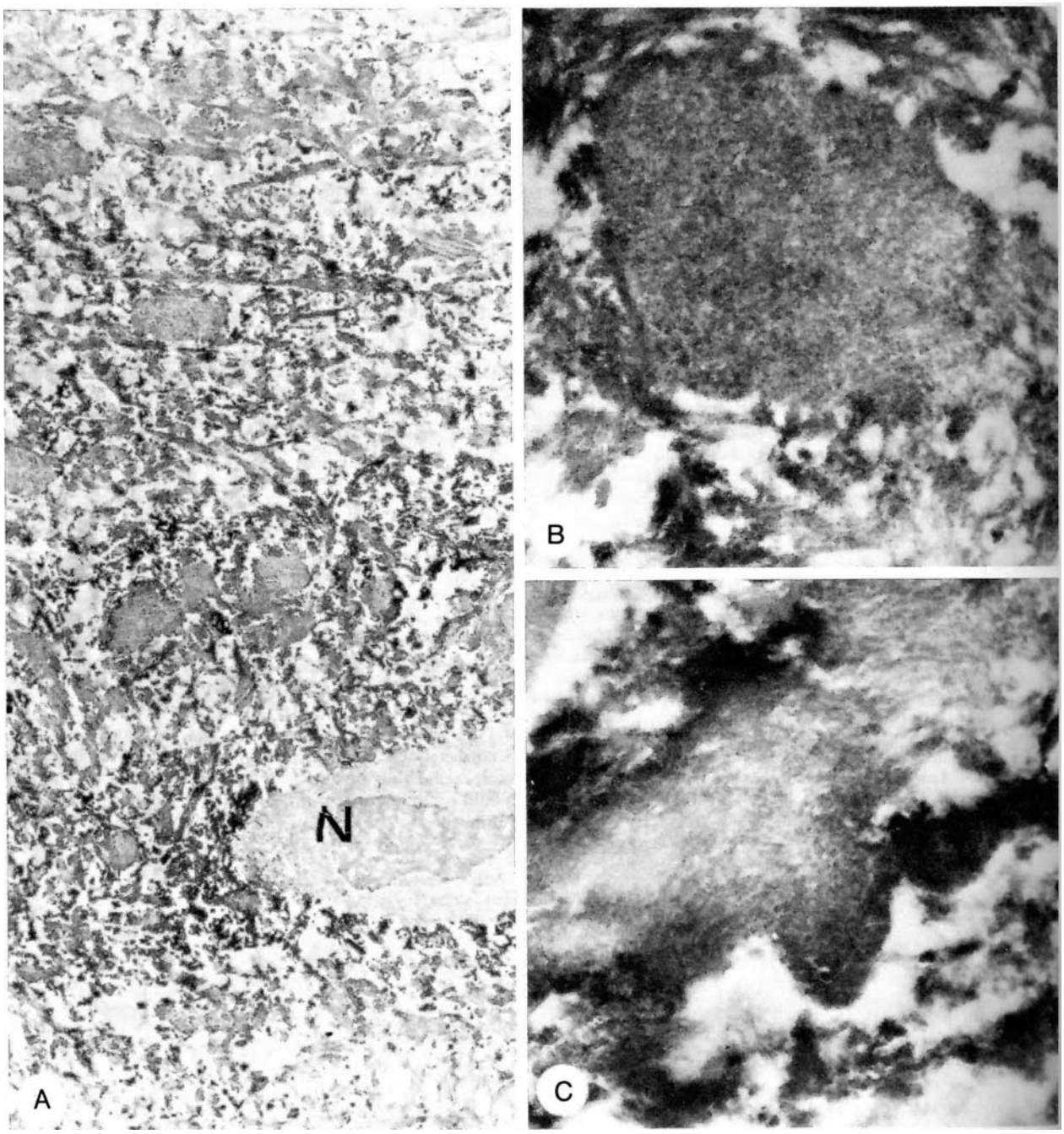

Figure 3. Electron microscopy of frozen sections of newborn rat skin extracted in 0.14 M NaCl in 0.1 M Tris-HCl, pH 8.0, stained with rabbit antikeratin IgG and peroxidase conjugated goat antirabbit IgG. Keratoliyalin granules and tonofilaments are stained *(A* × 7,500). An unstained nucleus *(N)* is seen. *B* in a higher magnification (× 44,000) of *A*. Poststain with uranyl acetate demonstrates filamentous nature of the internal structure of keratohyalin granules (*C* × 44,000). (From Fukuyama and Epstein, J Invest Dermatol, 74:179, 1980)

recognized in the large, dense keratohyalin of the transitional cell (see Fig 18) and was reflective of electron lucent filaments embedded within an electron dense matrix characteristic of the lower stratum corneum cells (see Fig 11d) [8].

Digestion of the dense substance of the KHG confirmed that filaments were an integral component (Fig 2). Extraction of skin with NaOH prior to embedding [9,15] or of thin sections of skin Tris-HCL buffered saline (TBS) [16], or 0.1 N HCl (human skin) post-embedding [17] unmasked filaments that were seen in sections by light and transmission electron microscopy and which reacted positively with an anti-keratin antibody by immunohistochemical staining. No reaction to the antibody could be demonstrated by KHGs in the tissue prior to extraction, indicating that the substance of the KHG prevented the antibody from recognizing the filaments [16] (Fig 3a–c).

Observation of the filament-keratohyalin relationship led to further speculation on the role of the KHGs in keratinization. Brody [14] suggested that the tonofilaments in the granular cell may become ''glued together'' by the substance of the KHG and converted directly to

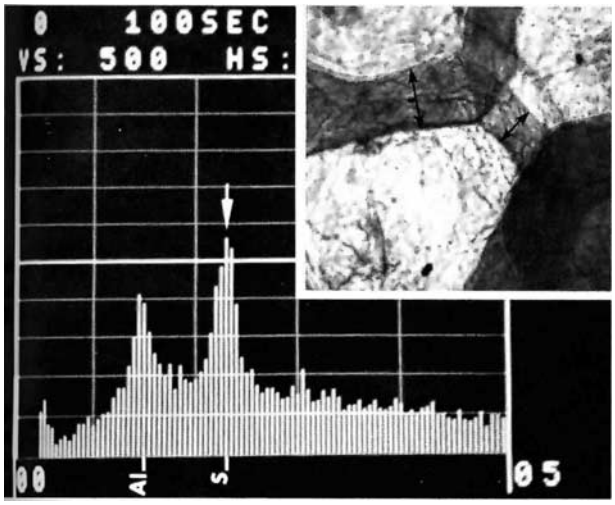

Figure 4. X-ray spectrum obtained for a sample of stratum corneum isolated tape stripping. The sample was analyzed without fixation by energy dispersive x-ray microanalysis using Cambridge Stereoscan Mark II Scanning Electron Microscope in the STEM Mode fitted with an ED AX 707A peak identification computer and CRT. A characteristic sulfur K_{alpha} peak recorded from the sample at the 2.3 keV position is significantly higher than the background radiation (continuous radiation in the spectrum shown to the right of the sulfur peak). The aluminum peak is the result of x-ray emission from the microscope chamber. The arrows indicate the overlap ftween squames of different layers.

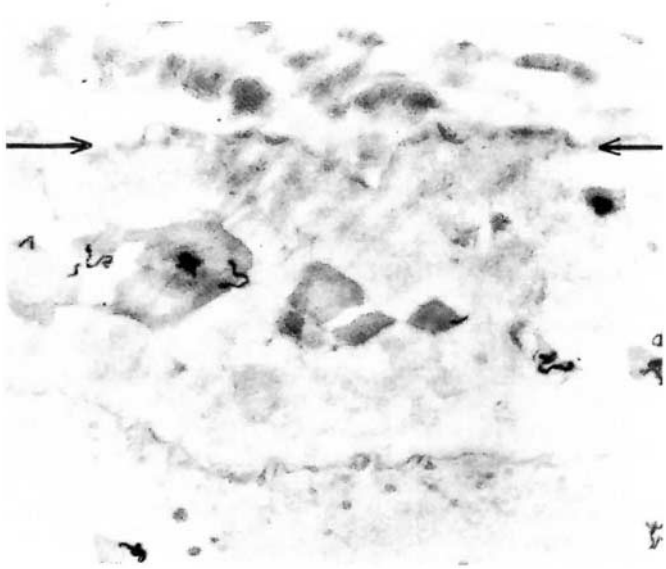

Figure 6. Radioautograph at the junction *(arrows)* between stratum granulosum and stratum corneum of newborn mouse epidermis two hours after intraperitoneal injection of Histidine-H^3. Portions of two cells contain keratohyaline granules (irregular gray bodies), upon some of which there are superimposed filamentous foci of reduced silver. Several silver particles do not correspond to keratohyalin granules, but none are seen in the stratum corneum (above the arrows). (× 8,300) (From Cox and Reaven, J Invest Dermatol, 49:32, 1967)

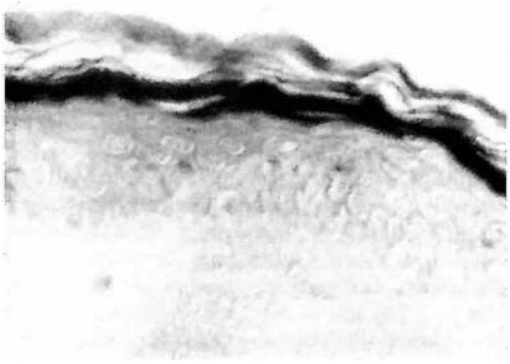

Figure 5. Normal human adult skin from abdomen showing concentration of histidine in transitional layers of epidermis after reaction with Pauly reagent. Formalin fixation. (× 365) (From Reaven and Cox, J Invest Dermatol, 45:424, 1965)

''keratin.'' Alternatively, he speculated that the tonofibrils might disintegrate or fragment to become the substance of keratohyalin which then would contribute, indirectly, to the formation of ''keratin'' [14]. The latter hypothesis was supported by farbman [18] and Bonneville [19]. We know today, from more complete characterization of the filamentous component and more precise knowledge of the molecular composition of the KHG, that the former hypothesis reflects more accurately the transition of keratin filaments and keratohyalin into the content of cells in the stratum corneum.

We have referred repeatedly to the electron dense content of the KGH as the ''substance'' of the KHG without defining it. Early attempts (using morphology) at understanding the composition were based largely on histochemical staining for specific organic molecules or functional groups and by digesting sections with enzymes that remove certain molecules selectively. Because of the noted coincidence of sulfur-contaimng ammo acids (cystine and cysteine) with keratinized tissue (stratum corneum, hair) and the presumed relationship of the keratohyalin granule with keratinization, sections of epidermis were stained for sulfur using a variety of 'methods which could distinguish between sulfhydryl groups and disulfide bonds. It was believed that sulfhydryl groups present on proteins within the granular layer were oxidized to disulfide bonds, creating strong protein linkages as cells moved into the cornified layers. It was not clear what proteins were involved in this process. The findings from these studies indicated, however, that while the granular layer—particularly the one most proximal to the stratum corneum—might be slightly more reactive with the histochemical staining for sulfur-containing compounds when compared with the other viable epidermal layers, the keratohyalin granules were clearly neither their primary source nor the site of deposition [20–27]. Staining of the stratum corneum cells for disulfide bonds was always enhanced over that of the viable epidermal cells, but the absence of positive sulfur staining of the KHGs in tissue suggested that this material was not involved in forming the disulfide linkages in cornified cells. High levels of sulfur in the stratum corneum could also measured by energy dispersive x-ray microanalysis (Fig 4). Autoradiographic studies were performed to follow the uptake and relocation of radiolabeled cystine in the epidermis. The granular layer showed a slight preference for the uptake of cystine [28], and there was some labeling of the KHG [27,29], but, confirming the histochemical data, the KHG was not the primary nor unique site where label was concentrated [29]. It should be noted, however, that isolated KHGs reacted positively with the histochemical stains for sulfur, suggesting that the reaction may be blocked *in vivo* [27]. We will discuss below that there is a population of small, sulfur-rich KHGs in rodent epidermis.

Keratohyalin granules were strongly and specifically stained by the Pauly reaction (diazotized sulfanilic acid), revealing the presence of histidine [30] (Fig 5). These observations corroborated biochemical

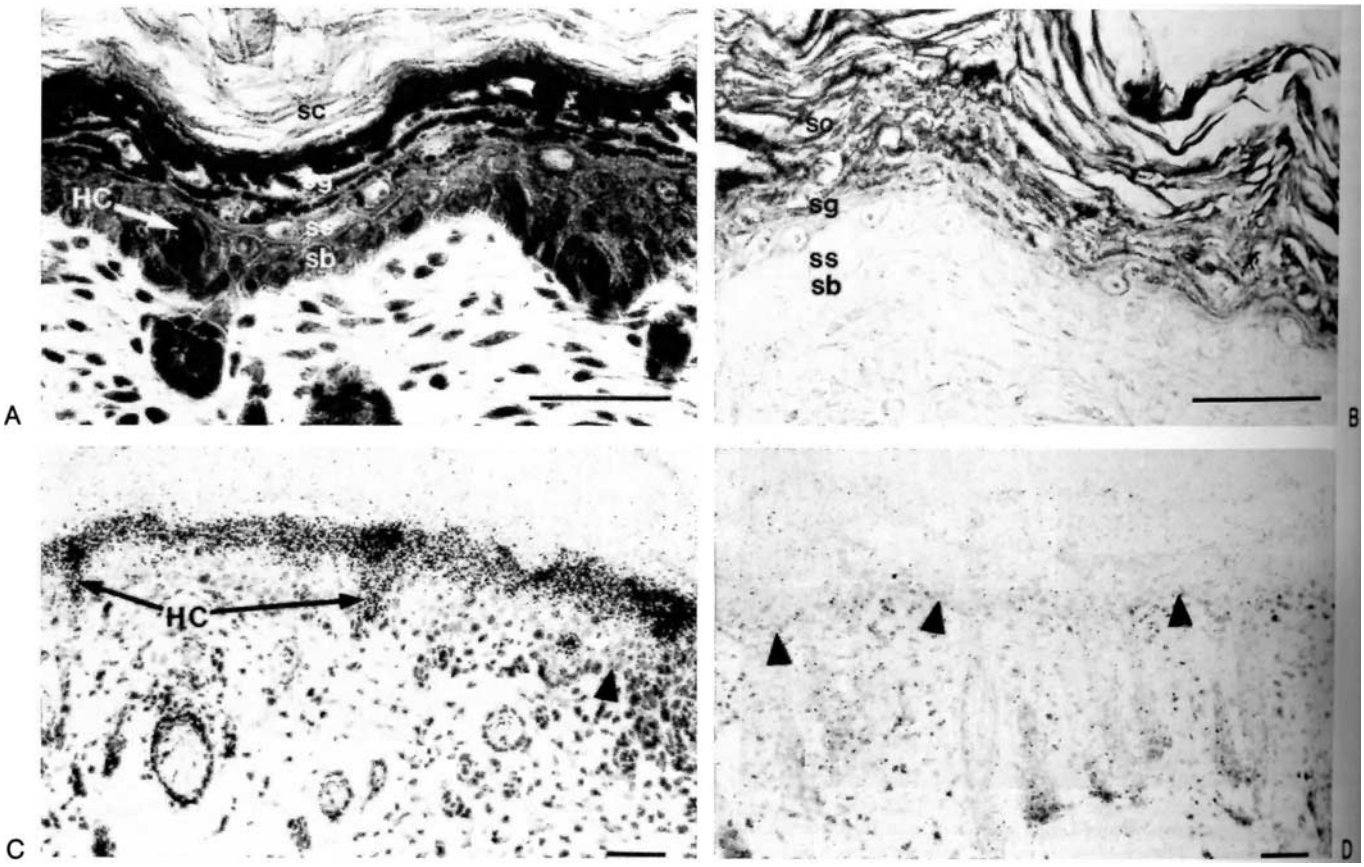

Figure 7. Paraffin sections of newborn rat skin stained with H and E or antifilaggrin/profilaggrin antibodies. *A,* Hematoxylin and eosin-stained paraffin section of newborn rat skin. *HC,* hair canals; *sb,* stratum basale; *ss,* stratum spinosum; *sg,* stratum granulosum; *sc,* stratum corneum. *Bar* = 50 μm. *B,* Localization of binding of antibodies directed against filaggrin/profilaggrin. Note reaction product in the granular cell layer. Abbreviations as *in A. Bar* = *50* μm. *C,* Bright-field micrographs of autoradiographs localizing hybridization of profilaggrin and plasmid (^{35}S) cRNAs. The profilaggrin cRNA probes hybridize in the granular cell layer and in the granular cells of the hair canals *(HC)* of the epidermis. *Arrowheads* indicate approximate location of the basement membrane. *Bar* = 50 μm. *D,* probes lacking profilaggrin sequences do not hybridize to sections of rat skin. *Arrowheads* indicate approximate location of the basement membrane. *Bar* = 50 μm. (From Fisher *et al*, J Invest Dermatol, 88:662,1987)

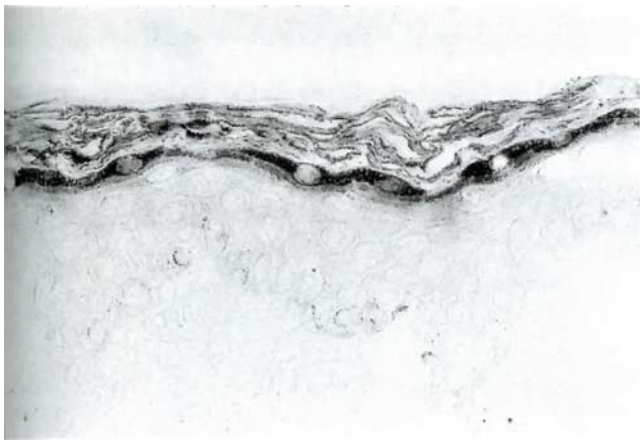

Figure 8. Immunohistochemical staining of adult epidermis with an antibody prepared against filaggrin from the stratum corneum. Note intense, granular stainng of the cells in the granular layer, as well as staining of the stratum corneum. × 400 (micrograph courtesy of Dr. Beverly Dale).

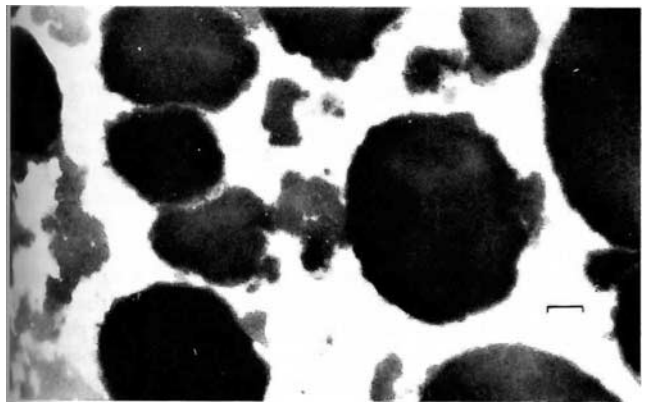

Figure 9. Isolated ''macroaggregates'' (KHG) which resemble KHG in situ. Marker: 0.2 micron (× 47,500) (From Sibrack *et al*, J Invest Dermatol, 62:397, 1974)

studies in which a sulfur-poor, histidine-rich, basic protein (now known as filaggrin) was identified in extracts of keratohyalin [31–34] and by labeling studies which showed that ^{3}H-histidine injected into animals (newborn rats) [35,36] or incubated with epidermal slices (cow snout) *in vitro* [37] was taken up rapidly, specifically and selectively by cells of the granular layer, localizing within the KHGs (Fig 6). Light and EM autoradiographs prepared from the tissue samples at several time points

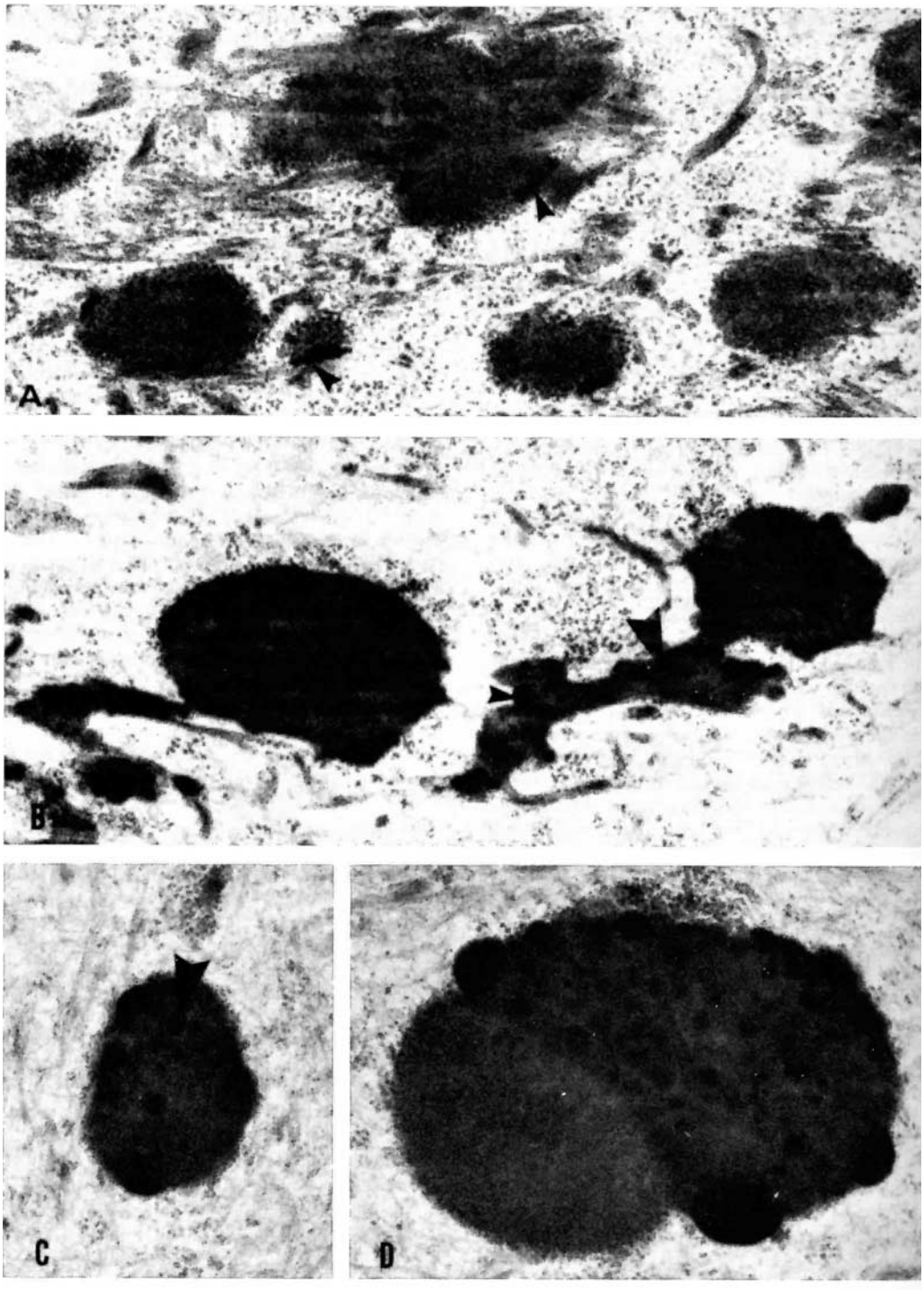

Figure 10. KG fixed in phosphate-buffered OsO_4 after fixing in glutaraldehyde. In the epidermis *(A)* DHD *(arrows)* are observed at the edge, often where attachment of tonofilaments are seen (× 30,000). In the transitional region *(B)* a combination of fibrillar component and DHD *(arrows)* compose a larger portion of KG, as compared to KG in the epidermis (× 30,000). In the buccal mucosa (C and *D)* DHD *(arrows)* appear in the center, as well as at the edge (× 30,000). (From Fukuyama and Epstein, J Invest Dermatol, 61:96, 1973)

after injection/incubation followed the labeled protein from the granular cells into the lower cornified cell layers and then to the outer layers of the stratum corneum [35,36]. Other labeled amino acids (glycine [38,39], arginine, serine and glycine [37,40]) were also incorporated into KHGs. This localization of histidine in the KHG and translocation through the cornified cell layer provided evidence that the KHG was an active site for new protein synthesis in the granular cell and that the protein synthesized was involved in the keratinization process. The messenger RNA for profilaggrin has been isolated, and a cDNA clone prepared against a 1200 bp fragment of a filaggrin cDNA clone has been used to

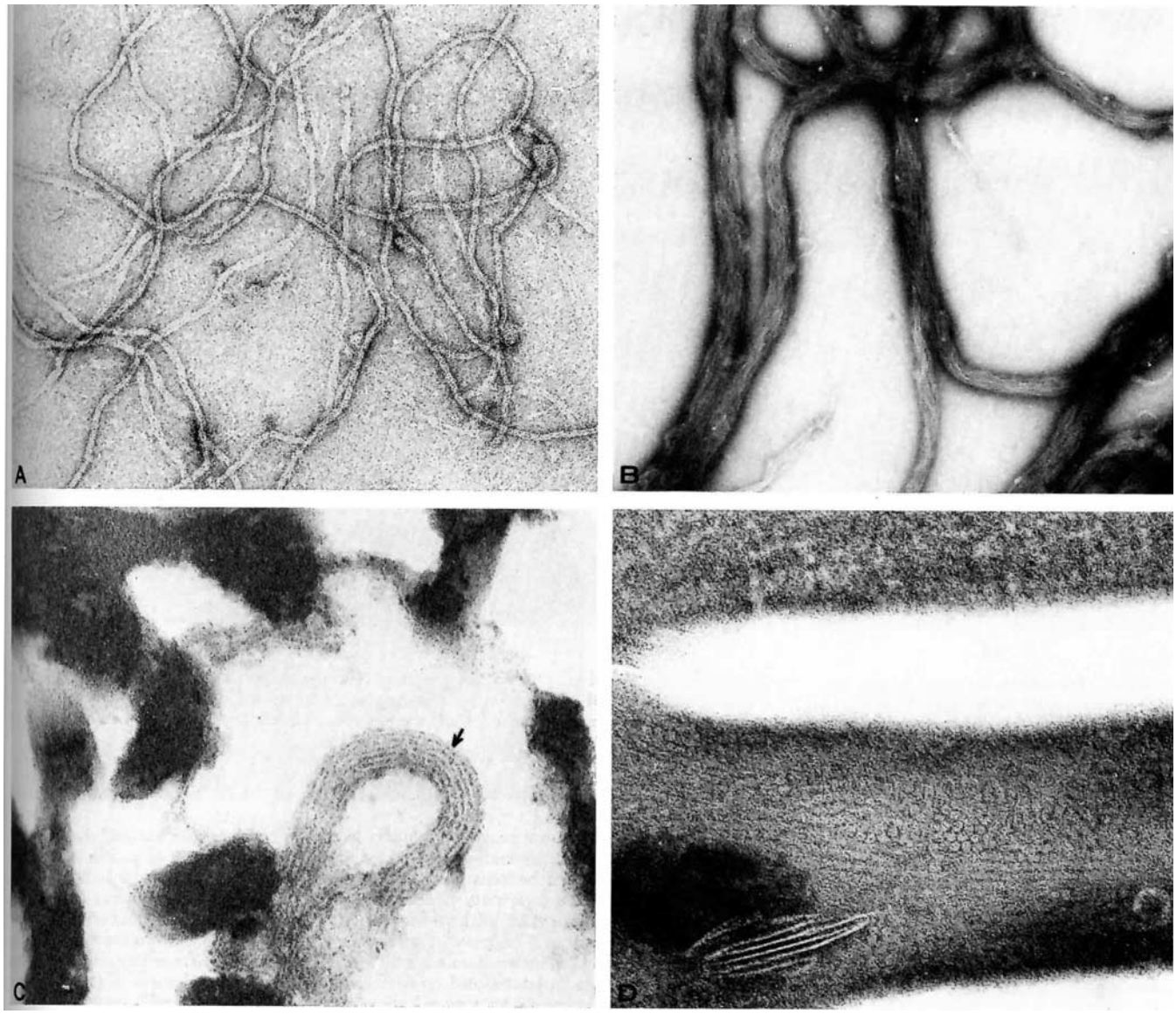

Figure 11. Transmission electron micrographs of *A)* reconstituted keratin filaments examined on an EM grid with negative staining, *B)* macrofibrils prepared by combining keratin intermediate filaments with keratohyalin-derived protein (stratum corneum basic protein, or filaggrin) *in vitro* and examining the preparations on an electron microscope grid with negative staining. Note that the individual filaments are consolidated into larger ropelike structures of regular diameter. C) Embedded, sectioned preparations of the macrofibrils and precipitated proteins (the electron dense material). Note the alignment of electron lucent filaments alternating with electron dense matrix within the macrofibril *(arrow). D)* Lower stratum corneum cell showing the keratin pattern of electron lucent filaments surrounded by electron dense matrix (presumably filaggrin). All micrographs $\times$ 58,500.

localize the message to the granular cells by *in situ* hybridization [41], thus demonstrating that the message for the keratohyalin-derived protein is synthesized in the same cell where it is translated (Fig 7a–d). Further studies have demonstrated the relationship of the protein in the granular and cornified layers.

Antibodies raised against the histidine-rich protein extracted from granular cells [42] and the stratum corneum [42–44] stained both KHG and cornified cells, indicating an immunologic homology between the proteins of these two layers (Fig 8). In concert with these observations were the biochemical data which revealed that histidine-rich, basic protein in the keratohyalin granule was a high molecule weight, phosphorylated, polymeric precursor (now called profilaggin [45]) of the immunologically cross-reactive protein in the stratum corneum cell [46–47].

Although most of the morphologic efforts to reveal the chemical composition of the KHG used tissue as starting material, morphologic techniques were also used to reveal the structure and composition of extracted keratohyalin granules reaggregated *in vitro* [48–50]. Phosphate-buffer extracted bovine [49] and rat [50] KHGs were centrifuged and dialyzed to form macroaggregates that were observed by scanning electron microscopy, or embedded for light and scanning and transmission electron microscopy (Fig 9) and reacted with several histochemical stains. The material in these preparations was found to be morphologically, histochemically [49–50] and immunologically [51] similar to that in the tissue.

Metallic ions have been observed to be associated with KHGs, either inherently or due to an affinitity of the protein within the granule for

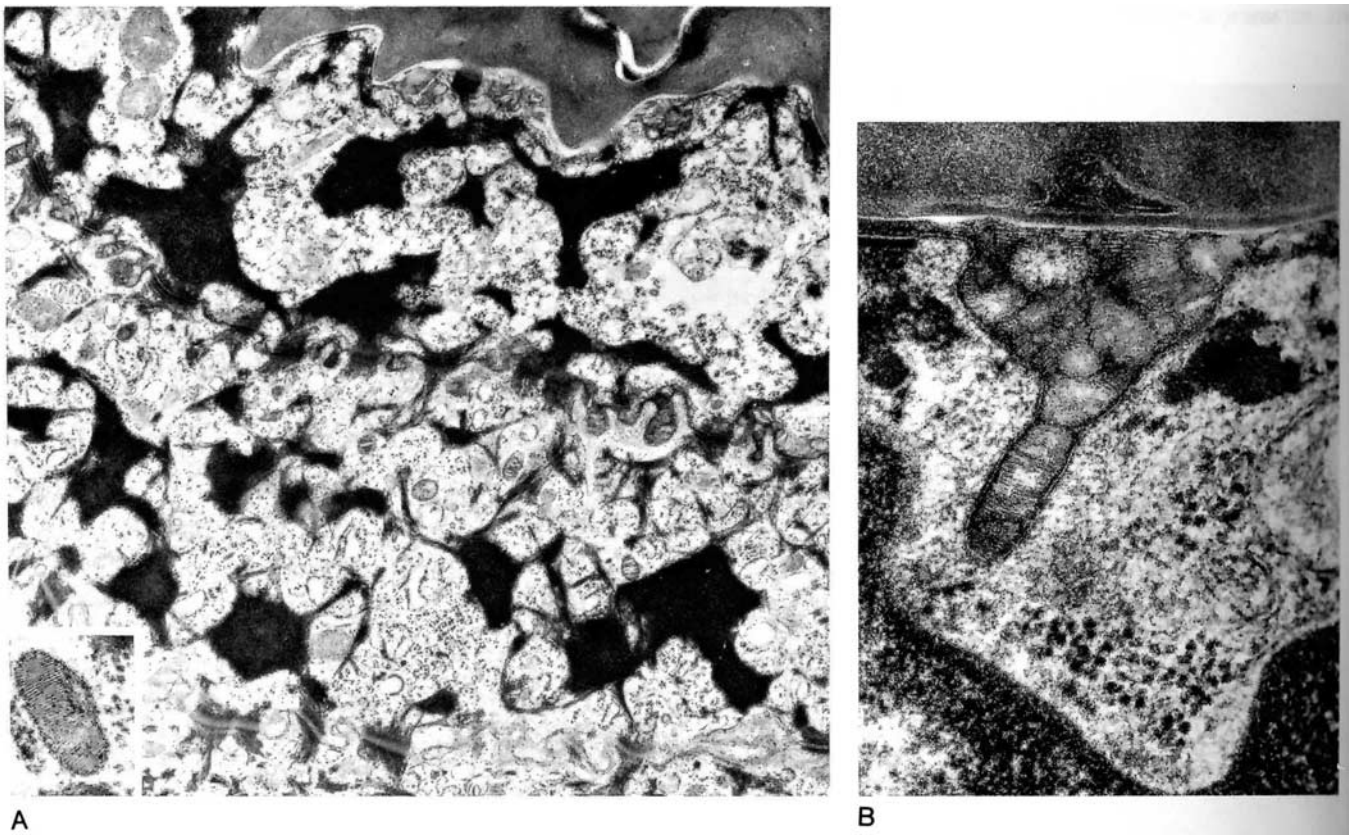

Figure 12. Transmission electron micrographs of lamellar granules in granular cells of human epidermis. Note the apparent relocation of granules toward the cell membrane in the upper granular layer cells *(A)*. The alternating dense lines in the interior of the granule are shown in the inset. A cluster of lamellar granules is seen in the intercellular space at the interface between the uppermost granular cell and the first cornified cell. *(A)* × 25,000; inset × 114,000; *(B)* × 108,750.

cations. The strong and selective binding of radiolabeled zinc seen in autoradiographs of the epidermis was shown to correlate the high histidine content of the KHG [52]. Binding of several other metallic ions was demonstrated by reacting tissue with metallic solutions, then recognizing the site of metal binding by the color of the staining reaction when the bound ion was chelated by unoxidized hematoxylin dye. The latter observations led to the speculation that the affinity of the KHGs for extraneous material may indicate that they have a role in binding and excreting toxic substances in the skin [53]. The more likely interpretation of their observations is that the ions bind to the phosphate groups on the profilaggrin or to ribonucleoprotein of the related ribosomes. X-ray microprobe analysis has been used to compare the elemental composition of keratohyalin granules and will be reviewed below in the context of the different components of the rodent KHG.

The KHGs of rodent epidermis vary in size, shape and chemical composition, according to location (Fig 10a–d). Those of the deeper layers are dense (osmiophilic), homogeneous and granular-appearing, and associated with ribosomes and filaments at their surfaces [9]. High resolution electron microscopic examination of this KHG has revealed a substructure composed of 200Å average diameter particles, sometimes organized in a lattice-like array [54]. The KHGs of this class are similar to those of the human.

Two types of KHGs are found in the uppermost granular cells. The first is the ''single granule'' [55], a small, round, uniformly dense KHG that is often located near the periphery of the cell and is not associated with filaments. Structures with the same morphology are also found in the nucleus of granular cells [55,56] and as a component of the second type of KHG (see below). In both the cytoplasm and the nucleus the single granules have been shown by x-ray microanalysis to be sulfur-rich [56] and by pepsin digestion of thin sections, proteinaceous [55]. Deposits of metallic silver over both the single granule and the cornified cell envelope in tissue which has been reacted with silver methenamine, and the position of the single granules adjacent to the cell membrane led Jessen [57] to propose that the single granules may be storage organelles for material of the cornified cell envelope. Subsequent studies (mentioned above) in which ^{3}H-cystine uptake and movement was followed in rodent epidermis did not support this relationship [29,36].

The second type of KHG in the upper granular layers is a larger, more-irregular and heterogeneous in structure ''composite'' granule [55,56] (Fig 9). The interior of the granule is dense and composed of a pepsin-sensitive, proteinaceous matrix [55]. Dense homogeneous deposits (DHD), suggested to be equivalent to the ''single granules,'' are embedded in peripheral, fibrillar material [9]. The composite granules are sulfur-poor, but generate a major phosphorous peak when analyzed by x-ray microprobe [56]. The high phosphorus content must correspond to the localization of profilaggrin.

We have alluded to a few potential functions of the keratohyalin granule in keratinization based on morphological evidence. Of these, the best supported, by all evidence, is the concept that the keratohyalin granule is a source of new protein (profilaggrin) which is processed to filaggrin during the granular cell-cornified cell transition. Within the cornified cell, filaggrin may function as an inter-filamentous matrix protein that aligns the keratin filaments, and hence functions as the ''interfilamentous glue'' described by Brody [14]. In 1970, Jessen [55] also proposed that the KHGs contribute to the interfilamentous material of the cornified cell and has shown recently that the KHG in glutaraldhyde-fixed tissue selectively bind colloidal gold-labeled staphylococcal protein A in the same manner as the lower stratum corneum

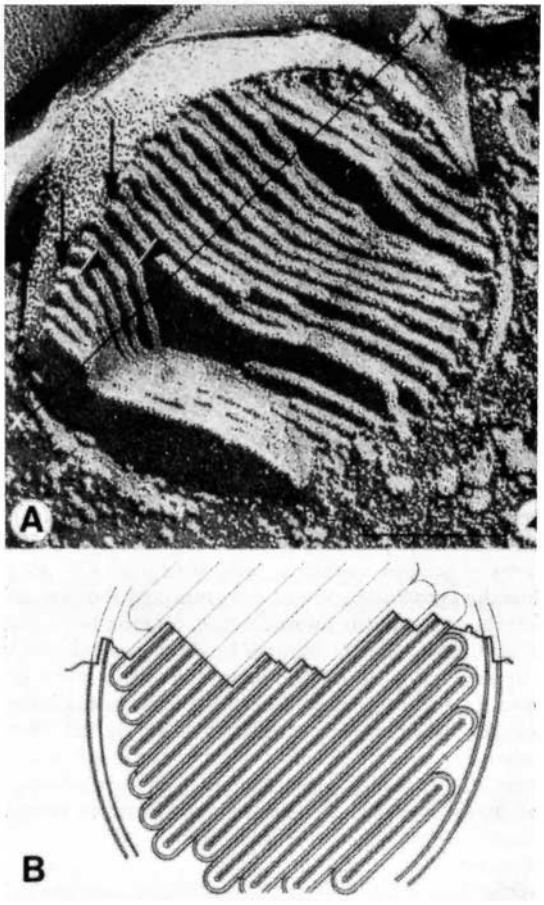

Figure 13. Lamellar granule disks in chicken MGBs *(A). B,* Profile of the fracture face traversing the MGB shown in *A* along the line X—X. Note wide fracture faces ending in a crescent deviating from the even cleavage plane, and intervening narrow faces *(arrows* and *arrowheads,* respectively, in *A.)* From Landmann, J Invest Dermatol, 87:204, 1986).

cells [58,59], thus suggesting similarity in the composition of these two sites. Breathnach [60] amplified this role, commenting that KHG substance could influence the extent of packing of filaments. Other lines of evidence reinforce a precursor-product relationship between the KHG and substance of the cornified cell.

More direct evidence for a role of the KHG-derived protein in filament aligment comes from experiments in which reconstituted keratin filaments in solution (Fig 11a) aggregated into macrofibrils when the keratohyalin-derived protein, filaggrin (but not profilaggrin) was added to the solution [61] (Fig 11b). When embedded in plastic, sectioned and examined by transmission electron microscope, the macrofibrils appeared as electron lucent filaments in dense matrix (Fig 11c), thus simulating the ''keratin pattern'' (Fig 11d).

Although the arguments for a role of keratohyalin-derived protein in providing the interfilamentous matrix in cornified cells and in aligning keratin filaments are compelling, the story is not yet complete because a ''keratin pattern'' can also be observed in stratum corneum cells of patients affected with ichthyosis vulgaris, a disorder characterized by the absence of keratohyalin granules and filaggrin [62]. These data indicate that the role of the keratohyalin granule is not fully resolved.

Lamellar Granules (Table II) Morphology was the basis for discovering the lamellar granule, and it has played a prominent role in unravelling its function. Lamellar granules are small (100–500 μm), membrane-bound organelles, named variously for the appearance of their contents (lamellar bodies, lamellar granules), their proported function in modifying the cell membrane during the granular-cornified cell transition (membrane coating granules), their relationship to keratinization (keratinosomes), or in recognition of some of the first investigators who described them (Odland bodies). They are found throughout the cytoplasm of upper spinous cells (often in relation to the Golgi [63]), increasing in number in granular cells where they eventually cluster adjacent to the cell membrane (Fig 12a,b). In the latter cells, they may account for 2% -15% of the volume [64].

Lamellar granules were first identified by electron microscopy as ''small granules'' that were believed to be degenerated or transformed mitochondria [65], viruses [11], secretory granules or vesicles (reviewed in [66]) or perhaps a unique class of keratohyalin granule. Structural similarities between the lamellar granule and mitochondria are frequently acknowledged in the literature, although lamellar granules are significantly smaller, typically found in different regions of the cell than the mitochondria, and differ markedly in chemical composition (see below). The comparison between the two organelles is based primarily on the similarities between the membrane structure of the mitochondrial cristae and the sheets (or tubules) in the interior of the lamellar granule [67].

Thin sections of epidermis examined by transmission electron microscopy at high magnification have been used to demonstrate alternating thick (20Å) and thin (10Å) dense lines separated by lighter lamellae of equal width (20A) (Fig 12a, inset) [68] within the lamellar granule, a morphology that is consistent with packing of a series of flattened disks or unilamellar liposomes within an outer, boundary membrane [69]. A model of this membrane structure has been prepared to explain the alternating ''lines'' seen within the granules when sectioned perpendicularly through the lamellae [69] (Fig 13). In some lamellar granules, all the lamellae are not parallel throughout, but are organized in two-three sectors, each of which contains of parallel lamellae [70,71].

The lamellar structure suggests that these organelles are rich in lipids, a presumption that has been confirmed by biochemical analysis [72–74] and by histochemical and cytochemical staining of isolated fractions of lamellar granules and/or tissue sections. Sections of epidermis reacted positively in the granular layer for phospholipids (demonstrated by acid hematin histochemical staining and by ictricomplex flocculation method for electron microscopy), glycolipids (periodic acid Schiff positive staining), and free sterols (digitonin-treated tissue prepared for freeze fracture) [75] (Fig 14).

Phospholipids were also demonstrated by digestion of the lamellar granules in thin sections with phospholipase C [76]. The reactions for glycoplipids and phospholipids were more intense in the granular cell cytoplasm than at the plasma membrane. In contrast, the membranes of stratum corneum cells were intensely positive for lipids, but the cytoplasm showed only minimal staining when the tissue was reacted with the fluorochrome, 8-anilino-1-naphthalene sulfonic acid, ANS, that recognizes lipids in general. Staining for neutral lipids (oil red O) was also strongly positive at the surface of the corneocytes, and free sterols were abundant in the intercellular spaces, as shown by freeze fracture images [75] (Fig 15). The change in histochemical staining of lipids between the granular and cornified layers corresponds to the biochemical data supporting a remodelling of the polar lipids derived from the lamellar granules into neutral lipids within the intercellular space, presumably by the activity of enzymes also contained within the lamellar granule (see below) [75].

Other organic molecules have also been demonstrated in the lamellar granule by their specific staining properties. As noted above, cabohydrate moieties, complexed with lipid or protein, were identified by positive staining with periodic acid Schiff reagent (light microscopy) [63] and periodic acid-thiocarbohydrazide silver protrminate (PA-TCH) (electron microscopy) [77] or periodic acid and bismuth (PA-Bi) [72]. Positive histochemical reactions also indicated that the lamellar granules contain several hydrolytic enzymes: acid phosphatase, carboxypeptidase,

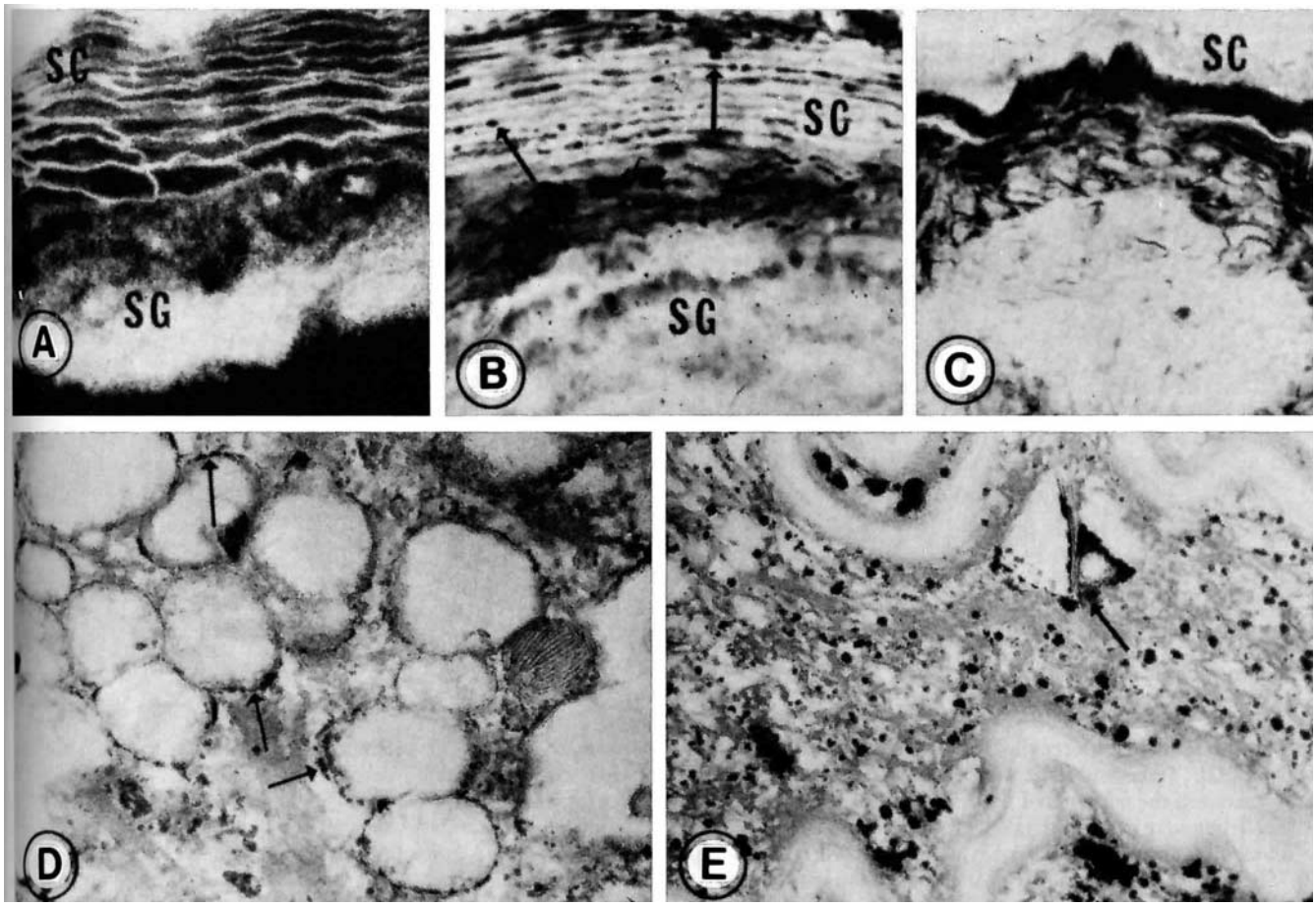

Figure 14. *A,* Unfixed frozen section of stratum granulosum plus stratum corneum stained with the fluorescent dye, 8-anilino-1-naphthalene-sulfonic acid (ANS). Note diffuse fluorescence of granular cells *(SG),* while fluorescence in stratum corneum *(SC)* is limited to membrane regions (× 800). *B,* unfixed, frozen section of stratum granulosum plus stratum corneum stained with oil red O. Note absence of stain in stratum granulosum *(SG),* while membrane regions of the stratum corneum *(SC)* are intensely stained. In some regions oil red O-stained material appears to form droplets or lens-like pockets *(arrows)* (× l,200). *C,* Formol-calcium fixed, frozen section stained with the Baker acid hematin method. Note intense staining throughout viable epidermis becoming minimal at the stratum corneum *(SC)*-stratum granulosum interface. The stratum corneum is not stained (× 450). *D* and *E,* Stratum granulosum *(D)* and stratum corneum *(E)* treated with the tricomplex flocculation method. In *D* note that the limiting membrane of lamellar bodies contains dense deposits *(arrows),* while the internal contents of these organelles is not blackened. In *E,* the cytoplasm of corneocytes contains scattered, fine granular deposits and dense aggregates *(arrows).* The latter appear adjacent to residual membrane-like spicules. Intercellular domains in both the stratum granulosum (not illustrated) and stratum corneum contain no deposits. *(D* × 95,000; *E* × 62,000). (From Elias *et al,* J Invest Dermatol, 73:342, 1979).

cathepsin B-like activity, *β*-gluronidase (low activity), aryl sulfatase A and B (low activity), acid lipase, steroid sulfatase, phospholipase A and sphingomyelinase [74,78–80], The lipases and glycosidases are thought to remodel the ''probarrier'' polar lipids (phospholipids and glycosphingolipids) into more hydrophobic, nonpolar products (ceramides and free fatty acids) that coalesce into sheets within the intercellular space forming an effective permeability barrier and facilitating desquamation [74,80]. The glycosidases also may act on glycoproteins of the keratinocyte surface (see below). The interdisk spaces of the lamellae within the granules and the intercellular lamellae appear to be storage sites for calcium ion, as demonstrated by the formation of electron dense precipiates when the tissue is reacted with oxalate-pyroantimonate-osmium [81]. Calcium in these locations may mediate the fusion and release of the contents of the lamellar granule into the intercellular space and promote their subsequent reoganization into lipid sheets [81].

The structure of the organelle itself is sufficiently unique to have attracted the attention of morphologists, but, in addition, the apparent movement of the lamellar granules toward the plasma membrane as cells move outward in the granular layer, their clustering at the membrane and fusion with the plasma membrane dispersing their contents into the intercellular space, and the similarity between the structure of their contents and the material in the intercellular space have led to obvious speculation about their function. It was thought initially that the released material coated the plasma membrane and thickened the border of the cornified cell (now known as the cornified cell envelope). Although biochemical studies have been crucial in dispelling this hypothesis by revealing the proteinaceous nature of the cornified cell envelope, and that its synthesis and deposition were submembranous rather than superficial, ultrastructural studies have also demonstrated that the contents of the lamellar granule were separated from the intracellular, dense material (thought to reinforce the membrane) by the plasma membrane [60,82]. Tracer substances that permeate the intercellular spaces contacted the contents of the lamellar granule on the *surface* of the cell, but were never seen taken into the cell or associated with the submembranous dense material of the cornified cell envelope [82]. These studies also argue against the hypothesis that lamellar granules form *from* invaginations of the cell membrane and move into the interior of the cell where they undergo dissolution—a mechanism once proposed

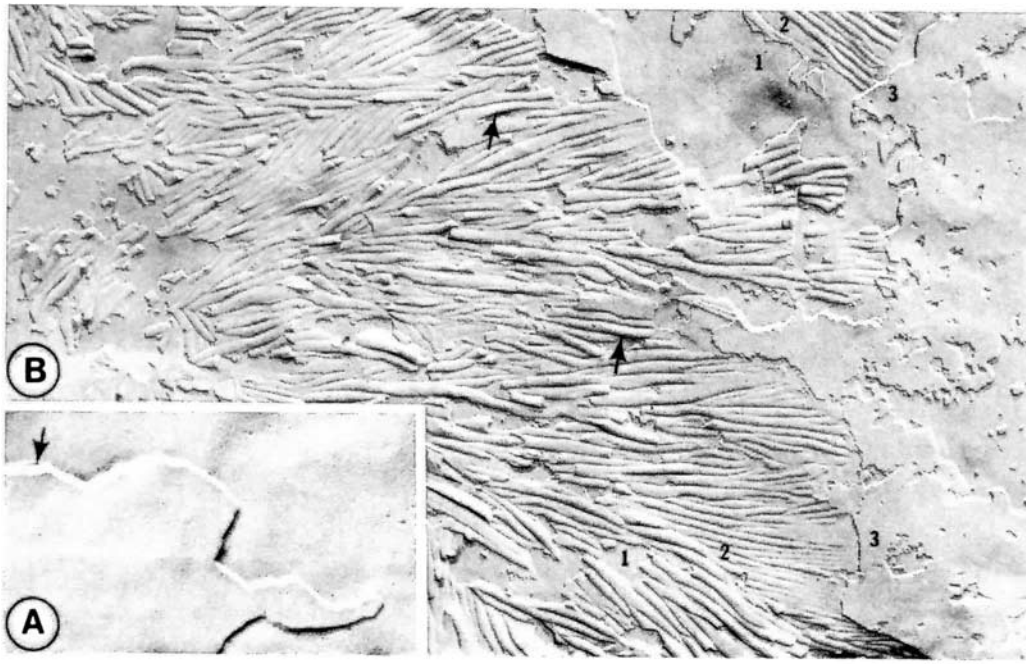

Figure 15. Stratum corneum intercellular domains before *(A)* and after *(B)* digitonin treatment. Note multilaminated nature of stratum corneum interstices *(indicated by numbers),* and the extensive com-plexing of free sterols in these regions *(arrows)* (A × 34,000; B × 56,500) (From Elias *et al*, J Invest Dermatol, 73:344, 1979.)

to explain reduction of the membrane surface area during the granular-cormfied cell transition [83].

It is now well understood that the contents of the lamellar granule are relased into the intercellular space where they reorganize into lamellae that are the structural basis for the permeability barrier in the epidermis [64,84]. Samples of skin prepared by freeze-fracture have been particularly effective in revealing the reorganized contents of lamellar granules, first into a series of discontinuous membranous disks that coalesce to form broad lipid sheets that fill the space between the granular and cornified cell [60,80,85–86]. Tracers (e.g., lanthanum, horseradish peroxidase and ferritin) injected Intradermally and allowed to percolate upward, or applied to the surface of the skin and allowed to penetrate into the deeper epidermal layers, were blocked (in either direction) at the position of the intercellular lipid lamellae; isolated, perfused epidermal sheets behaved in the same manner unless the tissue was pretreated with lipid solvents [64,86].

Information about the structure, function and composition of the lamellar granule has been acquired relatively recently and has unfolded in a more straight line course than was possible, for example, for the keratohyalin granule. Because these structures are submicroscopic, they eluded the histologists and were not subjected to intense investigation until a time when more-sophisticated techniques for study were available that could speed the progress in accumulating knowledge about this organelle.

Tonofilaments/Tonofibrils (Keratin Intermediate Filaments) (Table III) There are few areas in epidermal biology that rival the ''filaments'' (10 nm, keratin intermediate filaments) of keratinocytes for quantity of investigation at all levels—from pure description of their presence and organization to the molecular biology of the genes that encode their synthesis. Mention has already been made of the filaments in granular cells as they are related to the keratohyalin granule. This discussion, however, did not go beyond the statement of their presence or identification of their nature other than as their original, descriptive identity as ''tonofibrils.'' Currently we know a great deal about the biochemistry, regulation of synthesis, and patterns of expression of keratin filaments in different epidermal layers and under pathologic conditions. Morphology has *not* played a dominant role in these kinds of studies, but it has been useful to localize specific classes of filaments within different layers of cells and to reveal the substructure of the keratin filament.

The histology, ultrastructure and molecular structure of epidermal filaments have been investigated, in parallel, over the years. Ranvier described the anisotropic tonofibrils in the viable cells of the epidermis in 1879, but others thought these structures were either lymphatics, membrane artifact resulting from shrinkage (Herxheimer, 1889), or mitochondria (Parat, 1928) (reviewed in [4]). Filaments could not be seen in living cells that were stretched and teased apart [87] or even in the suboptimally fixed tissue prepared for electron microscopy; it was suggested that the previously reported images of filaments were gelated regions of cytoplasm [88,89]. Selby [90], however, clearly resolved filaments (named by her as ''tonofilaments'') in basal cells in thin-sectioned specimens of plantar epidermis and noted that they became ''consolidated'' into tonofibrils in the granular layer, assembled into a ''lacy network'' in the stratum compactum and then organized into an even more compact aggregation in the statum corneum (Fig 16). The filaments change in orientation from a basal-apical (vertical) distribution in basal cells to a perinuclear distribution in the spinous cells, then to a planar (horizontal) organization in granular cells, either effecting or conforming to changes in the major axis of the cell [14,91]. Selby concluded that the filaments in the cell persisted throughout keratinization, whereas other components of the cell were ''used up.'' The tonofilaments were precursors of ''keratin'' (as originally defined), and changes in their density and substructure implied changes in their molecular structure. Through these important morphologic observations, the continuity of the filaments throughout the epidermal layers and their apparent involvement in keratinization were established, concepts that were supported at the same time by x-ray diffraction analysis of extracted filament proteins, demonstrating that the x-ray patterns of tonofibrils in viable and cornified epidermal cells are the same [20,21,92–94].

Data on the structure and substructure of reconstituted keratin filaments (Fig 11a) formed under conditions of controlled polymerization

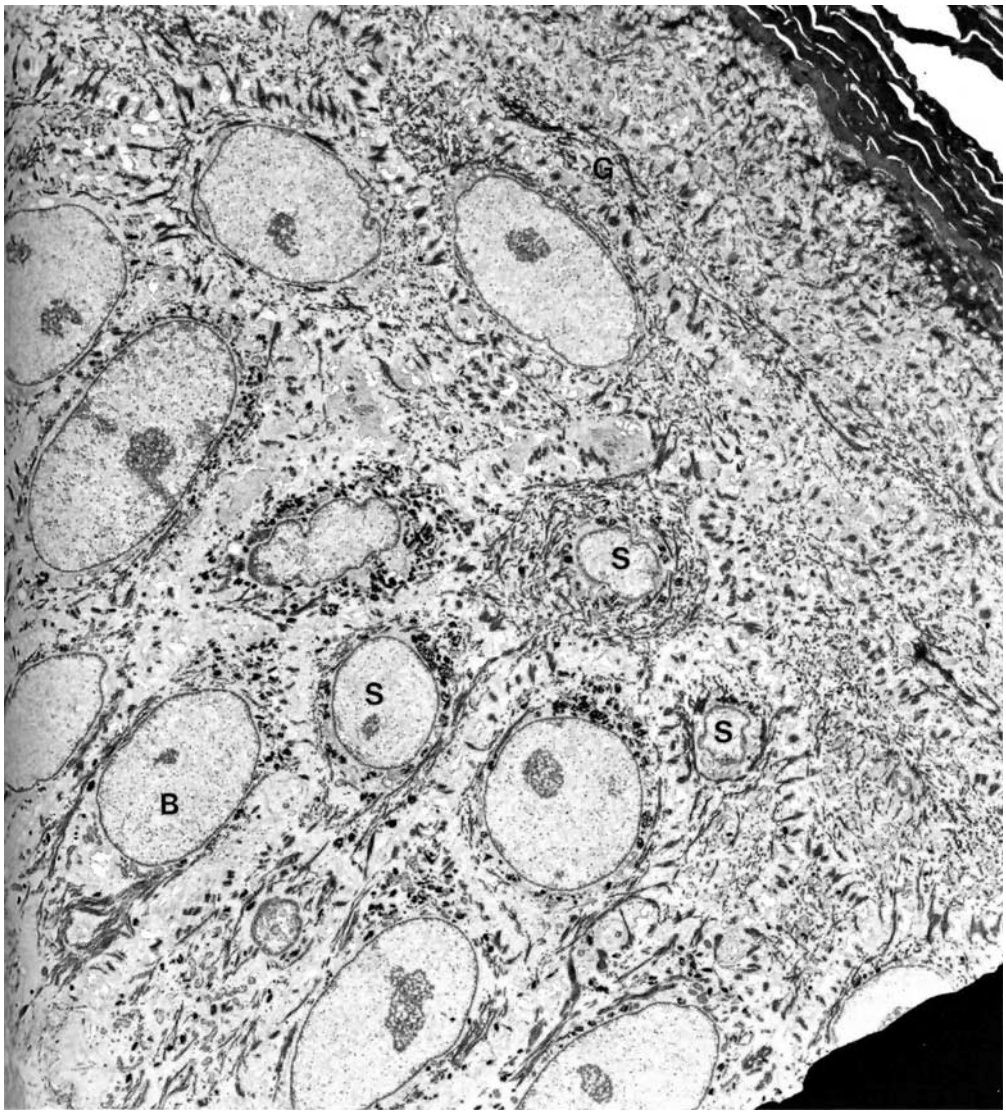

Figure 16. Transmission electron micrograph of full thickness adult epidermis showing the different organization of keratin filaments with cells of the basal, spinous and granular layers. Note the vertical orientation of filaments in basal cells *(B),* the perinuclear bundles of filaments in spinous layer cells (S) and the more vertical arrangement of filament bundles in the granular cells *(G)*. The filaments are associated with desmosomes in all cell layers. ×3675.

have been used to develop models of the molecular structure of the intermediate filament in general [95–97] and the keratin filament in particular [98]. Negatively stained preparations of the filaments revealed a substructure that consisted of four 4.5 nm diameter protofibrils, usually wound in a right-handed twist. The filaments were further unravelled when the filaments were reconstituted in phosphate buffer (or subsequently treated with phosphate buffer) at pH 6.0–6.5 into 2 nm protofilaments. An accurate number of these protofilaments could not be counted, but in optimally separated areas there appeared to be 6–8 strands per filament. Reconstituted filaments on grids were also subjected to proteolysis in various concentrations of trypsin; such preparations revealed portions of the filament that were unravelled, forming paracrystalline bundles which showed protofibrillar structure and along each of these a 5.4 nm axial repeat. The authors used these data to propose a model of the keratin filament to consists of protofilaments, perhaps assembled two or three stranded coiled-coil alpha helical segments of the keratin moolecule, into the four protofibrils which constitute the keratin filament. This model proposed for the keratin filament may represent the structure of other intermediate filaments as well [98].

How these filaments are involved in the keratimzation process has not come from the use of morphologic approaches, as much as it has from other work. Based on morphology, it was suggested that the filaments simply ''moved through'' the epidermis unchanged, serving as a scaffolding, a structural framework on which other events of keratinization could occur [12]. Kligman [99] described them as forming an ''endoskeleton'' that provides mechanical strength [100], yet permits elasticity and flexibility and, through their association with desmosomes, counterbalances distorsion on one part of the tissue [12]. Keratin filaments have also been suggested to help modulate and maintain cell shape, promote centration of the nucleus, and to implement cell-cell contact (via desmosomes) [101]. These observations support their obvious structural role in the cell, but do not address how they are involved in the process of keratinocyte differentiation leading ultimately to keratinization.

The relationship between tonofilaments/tonofibrils and ''keratin'' and the importance of these structures in keratinization has unfolded during the last 20 years largely through biochemical and molecular characterization of the proteins (a family of keratins) that comprise these structures. Morphology has played an important role in understanding this process

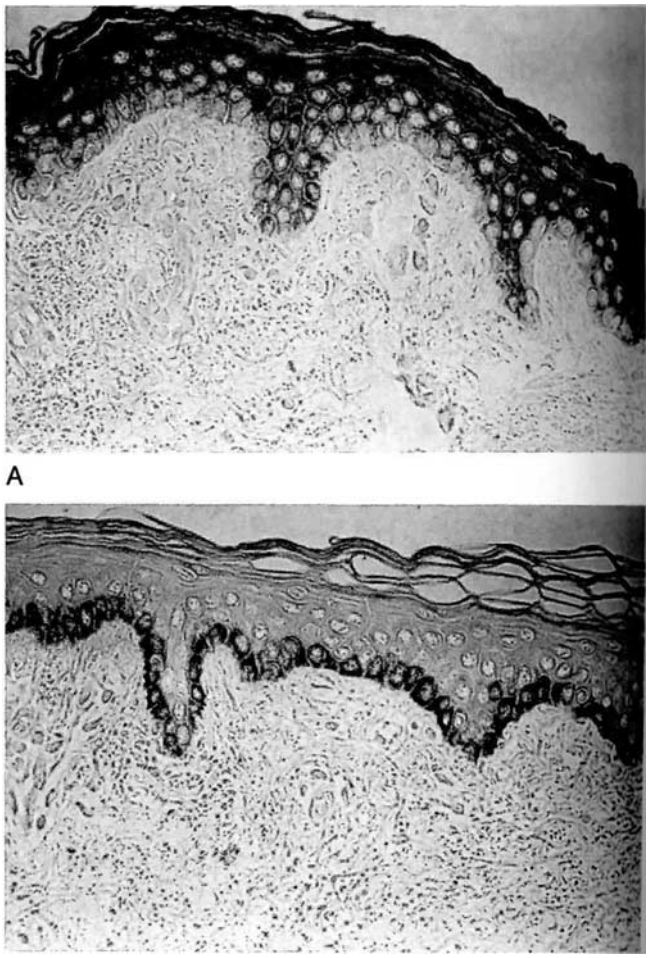

Figure 17. Immunohistochemical staining of keratin polypeptides in adult epidermis using the monoclonal antibodies AE2 *A* and AE1 *B* × 100 (on slide).

by revealing the localization of specific keratin species within different epidermal strata (and in other epithelia) and, in this regard, immunohistochemistry has been a particularly valuable technique.

All of the epidermal cells in keratinocyte cultures and epidermal sheets showed positive immunostaining when reacted with an anti-serum prepared against extracted, partially purified, total keratin from human callus [102]. The specific immunologic properties of individual keratins were revealed in later studies and became one of the criteria for recognizing heterogeneity among keratin filaments. Classes of keratins have been defined within the two subfamilies on the basis of charge, molecular weight, amino acid sequence, immunoreactivity and gene structure [103]. Keratins in mammalian epithelia fall into seven classes that can be recognized by monoclonal antibodies—in particular, the AE1, AE2 and AE3 monoclonal antibodies (prepared against keratins extracted from human callus that differ in molecular weight and charge) [104]. The use of these antibodies has increased our understanding of patterns of keratin expression in tissue according to state of differentiation [105], type of epithelium [104], body site, embryonic origin of the tissue [102], extrinsic conditions, disease state [106] and, to some extent, species [103]. Keratinizing epithelia contain the 65–67, 58, 56 and 50 kDa keratin species. Of these, the 65–67 kDa and 56 kDa keratins are found normally in suprabasal cells where they are recognized in immuno-stained preparations by a positive reaction with the AE2 monoclonal antibody (Fig 17a). The 50 and 58 kDa keratins are present in basal cells, recognized by positive immunoreactivity with the AE1

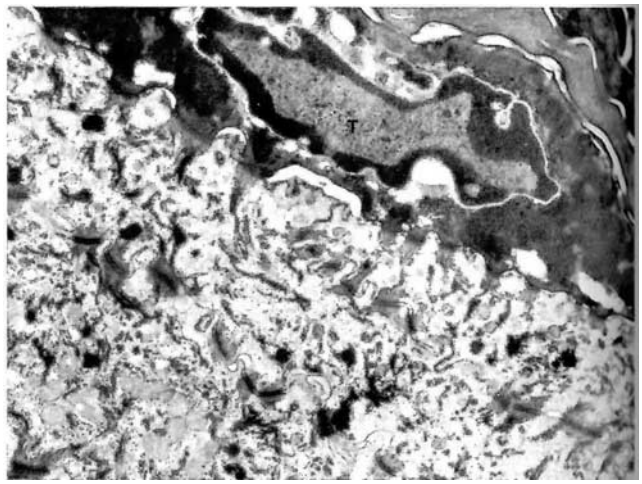

Figure 18. Transmission electron micrograph of a transitional keratinocyte *(T)* in adult epidermis. Note that the electron dense material in the cytoplasm is similar in morphology to the content of the first cornified cell. The nucleus is condensed and a cornified cell envelope is evident. A cluster or ribosomes is seen adjacent and inferior to the nucleus but no other organelles are apparent within the cell. × 17,500.

monoclonal antibody (Figs 17b). These keratins are also present in suprabasal cells (as demonstrated biochemically and by the use of other monoclonal antibodies that recognize a different epitope from the AE1 monoclonal [107,108], but the epitope recognized by AE1 is masked above the basal layer under normal conditions. This example illustrates the caution one must take in interpreting immunohistochemistry and demonstrates the importance of confirming negative results with other approaches [104].

Immunohistochemical staining of keratin filaments has also revealed the organization of filaments within cells and the relationships of keratin intermediate filaments with other cytoskeletal (microfilaments, microtubules) elements within the keratinocyte [109].

Granular Cornified Cell Transition: Degradative Events

The remarkable transition of the viable granular cell into the nonviable cornified cell is accomplished by the modification and transfer of some components between these layers (keratins, profilaggrin, enzymes, lipids and proteins of the cornified cell envelope), as well as by the degradation and loss of others (nucleus, cell organelles and ground substance). The latter is accomplished normally in tissue and in cultured keratinocytes without an accumulation of debris in the intercellular space. Observations made on ''transitional cells'' provide some insight into the structural changes that occur during this process (Fig 18). Transitional cells possess morphologic characteristics of both granular and cornified cells and, although they are seen only sporadically in sections of human epidermis, they form a continuous layer in the cow rumen. Ultrastructural studies of this layer have shown that such cells contain an increased number of lysosomes, autophagosomes containing lysed cell contents, aggregated KHGs, lipid accumulation, perinuclear clearing of the cytoplasm [10], and a cornified cell envelope [8]. Changes in the nucleus during this transition have been well-described.

Nuclei in the granular layer flatten and assume a horizontal orientation. Different images of their structure in transitional cells suggest that they go through phases of condensation and cessation of RNA synthesis, followed by degradation of the nucleoprotein, leaving nuclear remnants in the form of dense fibrillar material that is deposited outside of the nuclear envelope, presumably having been transported to that site [110]. Enzymes associated with the breakdown of DNA and RNA have been demonstrated biochemically [111], and areas of intense enzyme activity have been localized in the region of the upper granular cells and

lower stratum corneum by a method developed by Daoust [112] in which gelatin films containing DNA and RNA are applied to frozen sections of skin. At sites where the enzyme is present, the molecules in the film are digested, leaving clear areas in the stained film which can be superimposed on the stained section [113]. Studies of nuclear degradation in cultured keratinocytes have revealed that serum plasminogen activator plays an active role in nuclear destruction [114]. Digestion of thin sections of fixed, embedded tissue with RNase and pronase and staining for DNA with Schiff-thallium demonstrated that there is a decrease in RNA in the nucleolus, and not necessarily a correspondence between the electron dense material in the nucleus and the distribution of nucleic acid [115].

Movement of cells out of the granular layer is also associated with loosening of cells from one another and, ultimately, separation and desquamation. Because adhesion is brought about by desmosomes, cell-surface components and substances of the intercellular milieu, cell separation presumably results from changes in the same. The degradation of desmosomes begins in the granular layer and involves changes in both its structure and biochemistry [116]. Skerrow [117] demonstrated that the desmosomes of granular cells are more resistant to separation when the epidermis is treated with trypsin. The trypsin-resistant material may correspond to a 78-kDa glycopolypeptide that was identified by King *et al* [118] as a major concanavalin A-binding protein from extracted epidermis. An antibody made in response to this glycopolypeptide bound to material between the desmosomes in granular and lower cornified cell layers and cross-reacted with higher molecular weight Con-A binding glycoproteins, including desmoglein II/desmocollin thought to be involved in cell-to-cell adhesion [119]. The antibody failed to react with trypsin separated epidermis. The data were consistent with their interpretation that the smaller molecular weight glycopolypeptide is a breakdown product that arises from the desmosome during terminal differentiation, likely through the action of protease or glycosidase enzymes [118] that may be released from the lamellar granule. Lectin-binding studies and immunolabeling with antibodies against blood group antigens have also shown that progressive changes in the sugar moieties of cell-surface molecules (likely glycoproteins) accompany keratinocyte differentiation [120–124]. How these changes also may affect cell adhesion is unclear.

COMMENT

This is a cursory review of the recent history of the use of many morphologic techniques in skin biology, focusing on a discussion of the granular cell to demonstrate how morphology has been important in the evolution of understanding the structure and function of this cell. Descriptive information on cell structure has provided a framework used initially to identify the components in the cell and to formulate hypotheses about their role in epidermal function and differentiation. Morphology has also played a role in revealing data about the synthetic and degradative events that proceed simultaneously within this cell and about the molecular and ionic composition of its organelles. This information has been important throughout the years in the development of concepts of keratinization, although clearly there were major gaps in the morphologic data to be filled from research using other techniques to provide a solid basis for understanding this process.

While we have tried to emphasize the breadth of information that can be obtained from the array of morphologic techniques, and that the technologies of various disciplines overlap, contemporary research is successful in accelerating the pace in accumulating of new knowledge because of its highly interdisciplinary nature. The morphologist has an armamentarium of sophisticated, morphologic techniques to contribute to this collaboration, and further development of these tools should increases in pace with new approaches in other disciplines.

It was stated almost 40 years ago that''… the signs are unmistakable that the day of 'molecular biology is here'; the need for deeper structural knowledge that is met everywhere apparent in biological investigations is definitely beginning to be met'' in skin biology [125].

REFERENCES

1. Rothmans S: Presidential Address: Basic Research in dermatology. J Invest Dermatol 13:165–170, 1949
2. Braverman MS, Braverman IM: Three-dimensional reconstructions of objects from serial sections using a microcomputer graphics system: J Invest Dermatol 86:290–294, 1986
3. Mierowsky E, Behr G, Keys S: Some observations on keratohyalin in warts. Br J Dermatol 60:275–278, 1948
4. Montagna W: The cytology of mammalian epidermis and sebaceous glands. Int Rev Cytol 1:269–304, 1952
5. Selby CC: The fine structure of human epidermis of mammalian skin as revealed by electron microscopy. J Soc Cos Chem 7:584–599, 1956
6. Leuchtenberger C, Lund HZ: The chemical nature of the so-called keratohyaline granules of the stratum granulosum of skin. Exp Cell Res 2:150–152, 1951
7. Moberger G, De P: A cytochemical study of the cellular granules in the stratum granulosum of the epidermis. Exp Cell Res 8:578–582,1955
8. Brody I: An ultrastructural study on the role of the keratohyalin granule in the keratinization process. J Ultrastruct Mol Struct Res 3:84–104,1959
9. Fukuyama K, Epstein WL: Heterogeneous ultrastructure of the keratohyalin granule: a comparative study of adjacent skin and mucous membrane. J Invest Dermatol 61:94–100, 1973
10. Lavker RM, Matoltsy AG: Formation of horny cells: the fate of cell organelles and differentiation products in ruminal epithelium. J Cell Biol 144:501–512, 1970
11. Horstmann E, Knoop A: Elektronenmikroskopische studien an der Epidermis. I. Rattenpfote. Zeitschr f Zellforsch 47:348–362,1958
12. Charles A, Smiddy FG: The tonofibrils of the human epidermis. J Invest Dermatol 29:327–338, 1957
13. Charles A: An electron microscope study of cornification in the human skin. J Invest Dermatol 33:65–74, 1959
14. Brody I: The ultrastructure of the tonofibrils in the keratinization process of normal human epidermis. J Ultrastruct Res 4:264–297, 1960
15. Fukuyama K, Buxman MM, Epstein WL: The preferential extraction of keratohyalin granules and interfilamentous substances of the horny cell. J Invest Dermatol 51:355–364, 1964
16. Fukuyama K, Kakimi S, Epstein WL: Detection of a fibrous component in keratohyalin granules of newborn rat epidermis. J Invest Dermatol 74:174–180, 1980
17. Wolff K, Schreiner E: Differential enzymatic digestion of cytoplasmic components of keratinocytes: electron microscopic observation. J Ultrastruct Res 36:437–454, 1971
18. Farbman AI: Morphological variability in keratohyalin. Anat Rec 154:275–286, 1966
19. Bonneville MA: Observations on epidermal differentiation in the fetal rat. AmJ Anat 123:147–164, 1968
20. Derksen JC, Heringa GC, Weidinger A: On keratinization and cornification. Acta Neerland Morph 1:31–37, 1937
21. Rudall KM: The proteins of mammalian epidermis. Adv Protein Chem 7:253–290, 1952
22. Mescon H, Flesch P: Modification of Bennett's method for the histochemical demonstration of free sulfhydryl groups in skin. J Invest Dermatol 18:261–266, 1952
23. Eisen AZ, Montagna W, Chase HB: Sulfhydryl groups in the skin of mouse and guinea pigs. J Nat Cancer Instit 14:341–351, 1953
24. Barnnett RJ, Seligman AM: Histochemical demonstration of sulfhydryl and disulfide groups of protein. J Natl Cancer Institute 14:769–803, 1954
25. Montagna W, Eisen AZ, Rademacher AH, Chase HB: Histochemistry and cytochemistry of human skin. VI. The distribution of sulfhydryl groups and disulfide groups. J Invest Dermatol 23:23–32, 1954
26. Flesch P: Biochemical data in physiological and pathological epidermal keratinization. J Soc Cos Chem 7:521–530, 1956
27. Matoltsy AG, Lavker RM, Matoltsy MN: Demonstration of cystine containing protein in keratohyalin granules of the epidermis. J Invest Dermatol 62:406–410, 1974

28. Bern H, Harkness DR, Blair SM: Radioautographic studies on keratin formation. Proc Natl Acad Sci USA 41:55–60, 1972
29. Fukuyama K, Epstein WL: Sulfur-containing proteins and epidermal keratinization. J Cell Biol 40:830–838, 1969
30. Reaven EP, Cox AJ: Histidine and keratinization. J Invest Dermatol 45:422–431, 1965
31. Hoober JK, Bernstein IA: Protein synthesis related to epidermal differentiation. Proc Natl Acad Sci USA 56:594–601, 1966
32. Dale BA: Purification and characterization of a basic protein from the stratum corneura of mammalian epidermis. Biochim Biophys Acta 491:193–204, 1977
33. Ball RD, Walker GK, Bernstein IA: Histidine-rich proteins as markers of epidermis differentiation. J Biol Chem 252:5861–5868, 1978
34. Dale BA, Vadlamudi B, DeLap LW, Bernstein IA: Similarities between stratum corneum basic protein and histidine-rich protein II from newborn rat epidermis. Biochim Biophys Acta 668:98–106, 1981
35. Cox AJ, Reaven EP: Histidine and keratohyalin granules. J Invest Dermatol 49:31–34, 1967
36. Fukuyama K, Epstein WL: A comparative autoradiographic study of keratohyalin granules containing cystine and histidine. J Ultra-struct Res 51:314–325, 1975
37. Elias PM, Montague PM, Ugel AR: *In vitro* studies on the kinetics, composition, and homology of bovine keratohyalin. Exp Cell Res 73:95–100,1972
38. Fukuyama K, Nakamura T, Bernstein IA: Differentially localized incorporation of amino acids in relation to epidermal keratinization in the newborn rat. Anat Rec 152:525–536, 1965
39. Fukuyama K, Epstein WL: Epidermal keratinization: localization of istopically labeled amino acids. J Invest Dermatol 46:551–560, 1966
40. Ugel AR: Studies on isolated aggregating oligoribonucleoproteins of the epidermis with histochemical and morphological characteristics of keratohyalin. J Cell Biol 49:405–422, 1971
41. Fisher C, Haydock PV, Dale BA: Localization of profilaggrin mRNA in newborn rat skin by *in situ* hybridization. J Invest Dermatol 88:661–664, 1987
42. Murozuka T, Fukuyama K, Epstein WL: Immunochemical comparison of histidine-rich protein in keratohyalin granules and cornified cells. Biochim Biophys Acta 579:334–345, 1979
43. Dale BA, Ling SY: Immunologic cross-reaction of stratum corneum basic protein and a keratohyalin granule protein. J Invest Dermatol 72:257–261, 1979
44. Dale BA, Gown AM, Fleckman F, Kimball JR, Resing KA: Characterization of two monoclonal antibodies to human epidermal keratohyalin: reactivity with filaggrin and related proteins. J Invest Dermatol 88:306–313, 1987
45. Harding CR, Scott IR: Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. J Mol Biol 1709:651–673, 1983
46. Dale BA, Ling SY: Evidence of a precursor form of stratum corneum basic protein in rat epidermis. Biochem 18:3539–3546, 1979
47. Lonsdale Eccles JD, Haugen JA, Dale BA: A phosphorylated keratohyalin-derived precursor of epidermal stratum corneum basic protein. J Biol Chem 255:2235–2238, 1980.
48. Matoltsy AG, Matoltsy MN: The amorphous component of keratohyalin granules. J Ultrastruct Res 41:550–560, 1972
49. Ugel AR, Idler W: Further characterization of bovine keratohyalin. J Cell Biol 52:453–464, 1972
50. Sibrack LA, Gray RH, Bernstein IA: Localization of the histidine-rich protein in keratohyalin: a morphologic and macromolecular marker in epidermal differentiation. J Invest Dermatol 62:394–405, 1974
51. Guss SB, Ugel AR: Immunofluorescent antibodies to bovine keratohyalin and immunologic confirmation of homology. J Histochem Cytochem 20:97–106, 1972
52. Reaven EP, Cox AJ: The histochemical localization of histidine in the human epidermis and its relationship to zinc binding. J Histochem Cytochem 11:782–790, 1963
53. Pizzolato P, Lillie RD: Metal salts-hematoxylin staining of skin keratohyalin granules. J Histochem Cytochem 15:104–110, 1967
54. Lavker RM, Matoltsy AG: Substructure of keratohyalin granules of the epidermis as revealed by high resolution electron microscopy. J Ultrastruct Res 35:575–581, 1971
55. Jessen H: Types of keratohyalin granules. J Ultrastruct Res 33:95–115, 1970
56. Jessen H, Peters DT, Hall TA: Sulfur in epidermal keratohyalin granules: a quantitative assay by x-ray microanalysis. J Cell Sci 22:161–171,1976
57. Jessen H: Electron cytochemical demonstration of sulfhydryl groups in keratohyalin granules and in the peripheral envelope of cornified cells. Histochemie 33:15–19, 1973
58. Jessen J, Behnke O: Selective binding of colloidal gold-protein conjugates to epidermal phosphorus-rich keratohyaline granules and cornified cells. J Invest Dermatol 87:737–740, 1986
59. Haftek M, Chignol M-C: Postembedding immunogold labeling of epidermis. J Invest Dermatol 90:87–88, 1988
60. Breathnach AS: Aspects of epidermal ultrastructure. J Invest Dermatol 65:2–15, 1975
61. Dale BA, Holbrook KA, Steinert PM: Assembly of stratum corneum basic protein and keratin filaments into macrofibrils. Nature 276:729–731, 1978
62. Sybert VP, Dale BA, Holbrook KA: Ichthyosis vulgaris: identification of a defect in synthesis of filaggrin correlated with an absence of keratohyaline granules. J Invest Dermatol 84:191–194, 1985
63. Matoltsy AG, Parakkal PF: Membrane-coating granules of keratinizing epithelia. J Cell Biol 24:297–307, 1965
64. Elias PM, Friend DS: The permeability barrier in mammalian epidermis. J Cell Biol 65:180–191, 1975
65. Selby CC: An electron microscope study of epidermis of mammalian skin in thin sections. II. Superficial cell layers of footpad epidermis. J Invest Dermatol 29:131–150, 1957
66. Matoltsy AG: Membrane-coating granules of the epidermis. J Ultrastruct Res 15:510–515, 1966
67. Odland GF: Submicroscopic granular component in human epidermis. J Invest Dermatol 34:11–15, 1960
68. Martinez, Jr, IR, Peters A: Membrane-coating granules and membrane modifications in keratinizing epithelia. Am J Anat 130:93–120, 1971
69. Landmann L: Epidermal permeability barrier: transformation of lamellar granule-disks into intercellular sheets by a membrane-fusion process, a freeze-fracture study. J Invest Dermatol 87:202–209,1986
70. Frithiof L, Wersall J: A highly ordered structure in keratinizing human oral epithelium. J Ultrastruct Res 12:371–379,1965
71. van de Staak WJBM, Stadhouders AM, Gilsing H: A comparative electron microscopic and histochemical investigation of membrane-coating granules in normal human skin and in the skin of psoriasis vulgaris patients. 180th Meeting Netherl Soc Derm (Leiden), 1968. Dermatologica 138:341–345, 1969
72. Freinkel RK, Traczyk TN: A method for partial purification of lamllar granules from fetal rat epidermis. J Invest Dermatol 77:478–482, 1981
73. Wertz PW, Downing DT, Freinkel RK, Traczyk TN: Sphingolipidi of the stratum corneum and lamellar granules of fetal rat epidermis. J Invest Dermatol 83:193–195, 1984
74. Grayson S, Johson-Winegar A, Wintroub BU, Isseroff RR, Epstein EH Jr, Elias RM: Lamellar body-enriched fractions from neonaul mice: preparative techniques and partial characterization. J Invest Dermatol 85:289–294, 1985
75. Elias PM, Brown BE, Fristch P, Goerke J, Gray M, White RJ: Localization and composition of lipids in neonatal mouse stratum granulosum and stratum corneum. J Invest Dermatol 73:339–348. 1979
76. Wolff-Schreiner E: Ultrastructural cytochemistry of the epidermis Int J Dermatol 16:77–102, 1977

77. Hayward AF, Hackemann M: Electron microscopy of membrane-coating granules and a cell surface coat in keratinized and nonkeratinized human oral epithelium. J Ultrastruct Res 43:205–219, 1973

78. Wolff K, Holubar K: OdlandKorper (membrane-coating granules. keratinosomen) als epidermale lysosomen. Arch Klin Exp Dermatol 231:1–19, 1967

79. Freinkel RK, Traczyk TN: Acid hydrolases of the epidermis: subcellular localization and relationship to cornification. J Invest Dermatol 80:441–446, 1983

80. Elias PM, Menon KG, Grayson S, Brown BE: Membrane structural alterations in murine stratum corneum: relationship to the localization of polar lipids and phospholipases. J Invest Dermatol 91:3–10, 1988

81. Menon G, Grayson S, Elias P: Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. J Invest Dermatol 48:508–512, 1985

82. Hashimoto K: The marginal band: a demonstration of the thickened cellular envelope of the human nail with the aid of lanthanum staining. Arch Derm 103:387–393, 1971

83. Rupee M, Braun-Falco O: Zur Ultrastruktur un Genese der intracytoplasmatischen Korperchen in normaler menschlicher Epidermis. Arch Klin Exptl Dermatol 221:184–193, 1965

84. Elias PM, Brown BE: The mammalian cutaneous permeability barrier: Defective barrier function in essential fatty acid deficiency correlates with abnormal intercellular lipid deposition. Lab Invest 39:574–583, 1978

85. Breathnach AS, Goodman T, Stolinski C, Gross M: Freeze-fracture replication of cells of stratum corneum of human epidermis. J Anat 114:65–81, 1973

86. Elias PM, McNutt NS, Friend DS: Membrane alterations during cornification of mammalian squamous epithelia: a freeze-fracture, tracer, and thin-section study. Anat Rec 189:577–594, 1977

87. Chambers R, Renyi GS: The structure of the cells in tissues as revealed by microdissection. I. The physical relationships of the cells in epithelia. Anat Rec 24:1–19, 1925

88. Pease DC: Electron microscopy of human skin. Am J Anat 89:469–498, 1951

89. Adolph WE, Baker R, Leiby GM: Electron microscope study of epidermal fibers. Science 113:685–686, 1951

90. Selby CC: An electron microscope study of epidermis of mammalian skin in thin sections. I. Dermo-epidermal junction and basal cell layer. J Biophys Biochem Cytol 1:429–444, 1955

91. Roe DA: Further studies of a fibrous keratin precursor from the human epidermis. J Invest Dermatol 27:319–324, 1956

92. Giroud A, Champetier G: Recherches sur les reontgenogrammes des keratines. Bull Soc Chim Biol 18:656–664, 1936

93. Astbury WT, Woods HJ: The x-ray interpretation of the structure and elastic properties of hair keratin. Nature 126:913–914, 1930

94. Carruthers C, Woernley DL, Baumler MS, Kress B: Proteins of mammalian epidermis. J Invest Dermatol 25:89–101, 1955

95. Steinert PM, Idler WW: Self-assembly of bovine epidermal keratin filaments *in vitro*. Mol Biol 108:547–567, 1976

96. Steinert PM, Idler WW, Goldman RD: Intermediate filaments of baby hamster kidney (BHK-21) cells and bovine epidermal kerati-nocytes have similar ultrastructures and subunit domains. Proc Natl Acad Sci USA 77:4534–4538, 1980

97. Steven AC, Hainfeld JF, Trus BL, Wall JS, Steinert PM. Ths distribution of mass in heteropolymer intermediate filaments assembled *in vitro*. J Biol Chem 258:8323–8329, 1983

98. Aebi U, Fowler WE, Rew P, Sun T-T: The fibrillar substructure of keratin filaments unravelled. J Cell Biol 97:1131–1143, 1983

99. Kligman AM: The biology of stratum corneum. In: Montagna W, Lobitz WC (eds.), The Epidermis. New York, Academic Press, 1964, pp 387–433

100. Matoltsy AG, Odland GF: Observations on the orientation of keratin in thickened cornified epithelium of the human skin. J Invest Dermatol 26:121–126, 1956

101. Jones JCR, Goldman AE, Steinert PM, Yuspa S, Goldman RD: Dynamic aspects of the supramolecular organization of intermediate filament networks in cultured epidermal cells. Cell Motility 2:197–213, 1982

102. Sun T-T, Green H: Immunofluorescent staining of keratin fibers in cultured cells. Cell 14:469–476, 1978

103. Cooper D, Sun T-T: Monoclonal antibody analysis of bovine epithelial keratins. J Biol Chem 261:4646–4654, 1986

104. Woodcock-Mitchell J, Eichner R, Nelson WG, Sun T-T: Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. J Cell Biol 95:580–588, 1982

105. Tseng SCG, Jarvinen MJ, Nelson WG, Huang J-W, Woodcock-Mitchell J, Sun T-T: Correlation of specific keratins with different types of epithelial differentiation: Monoclonal antibody studies. Cell 30: 361–372, 1982

106. Weiss RA, Guillet GYA, Freedberg IM, Farmer ER, Small EA, Weiss MM, Sun T-T: The use of monclonal antibody to keratin in human epidermal disease: alterations in immunohistochemical staining pattern. J Invest Dermatol 81:224–230, 1983

107. Hashimoto K, Eto H, Matsumoto M, Hori K: Anti-keratin monoclonal antibodies: production, specificities and applications. J Cutan Pathol 10:529–539, 1983

108. Eto H, Hashimoto K, Kobayashi H, Matsumoto M, Kanzaki T, Mehregan AH, Weiss RA: Monoclonal antikeratin antibody: production, characterization, and immunohistochemical application. J Invest Dermatol 84:404–409, 1985

109. Osborn M, Born T, Koitsch H-J, Weber K: Stereo Immunofluorescence Microscopy: I. Three-dimensional arrangement of microfil-aments, microtubules and tonofilaments. Cell 14:477–488, 1978

110. Karasek J: Nuclear morphology of transitional keratinocytes in normal human epidermis. J Invest Dermatol 91:243–246, 1988

111. Santoiana P, Rothman S: Nucleic acid splitting enzymes in human epidermis and their possible role in keratinization. J Invest Dermatol 37:489–495, 1961

112. Daoust R, Amano H: The localization of ribonuclease activity in tissue sections. J Histochem Cytochem 8:131–134, 1960

113. Steigleder GK, Raab WP: The localization of ribonuclease and deoxyribonuclease activities in normal and psoriatic epidermis. J Invest Dermatol 37:209–214, 1962

114. Green H: Terminal differentiation of cultured human epidermal cells. Cell 11:405–416, 1977

115. Suzuki H, Fukuyama K, Epstein WL: Changes in nuclear DNA and RNA during epidermal keratinization. Cell Tiss Res 184:155–167,1977

116. Allen TD, Potten CS: Desmosomal form, fate and function in mammalian epidermis. J Ultrastruct Res 54:94–105, 1975

117. Skerrow CJ: The experimental production of high-level intraepidermal splits. Br J Dermatol 102:75–83, 1980

118. King IA, Tabiowo A, Fryer PR: Evidence that major 78–44-kD Concanavalin A-binding glycopolypeptides in pig epidermis arise from the degradation of desmosomal glycoproteins during terminal differentiation. J Cell Biol 105:3053–3063, 1987

119. Cowin P, Mattey D, Garrod D: Identification of desmosome surface components (desmocollins) and inhibition of desmosome formation by specific FAB'. J Cell Sci 70:41–60, 1984

120. Brabec RK, Peters BP, Bernstein IA, Gray RH, Goldstein IJ: Differential lectin binding to cellular membranes in the epidermis of the newborn rat. Proc Natl Acad Sci USA 77:477–479, 1980

121. Nemanic MK, Whitehead JS, Elias PM: Alterations in membrane sugars during epidermal differentiation. J Histochem Cytochem 31:887–897, 1983

122. Reano A, Faure M, Jacques Y, Reichert U: Use of lectins for the study of human epidermal differentiation. Br J Dermatol 107, Suppl 23: 143–146,1982

123. Virtanen I, Kariniemi A-L, Holthofer H, Lehto V-P: Fluorochrome-coupled lectins reveal distinct cellular domains in human epidermis. J Histochem Cytochem 34:307–315, 1986

124. Dabelsteen E, Buschard K, Hakomori S-I, Young WW: Pattern of distribution of blood group antigens on human epidermal cells during maturation. J Invest Dermatol 82:13–17, 1984
125. Astbury WT: The molecular structure of skin, hair and related tissues. Br J Dermatol Syph 62:1–15, 1950
126. Christophers E, Braun-Falco O: Fluorescein-isothiocyanate as a stain for keratinizing epithelia. Arch Derm Forsch 241:199–209, 1971
127. Huff JC, Weston WL, Wanda KD: Enhancement of specific immunofluorescent findings with use of a para-phenylenediamine mounting buffer. J Invest Dermatol 78:449–450, 1982
128. Menton DN, Eisen AZ: Structure and organization of mammalian stratum corneum. J Ultrastruct Res 35:247–264, 1971
129. Roe DA: Polarization optical studies of hyperkeratosis, parakeratosis and dyskeratosis. J Invest Dermatol 33:257–266, 1959
130. Olerud JE, O'Brien, Jr, W, Reiderer-Henderson MA, Steiger D, Forster K, Daly C, Ketterer DJ, Odland GF: Ultrasonic assessment of skin and wounds with the scanning laser acoustic microscope. J Invest Dermatol 88:615–623, 1987
131. Odland GF: The fine structure of the interrelationship of cells in the human epidermis. J Biophys Biochem Cytol 4:529–538, 1958
132. Runge W, Younger B, Zelickson AS: Optical transforms of epidermal Langerhans' cell granules. J Invest Dermatol 77:421–429, 1981
133. Forslind B, Wroblewski R, Afzelius BA: Calcium and sulfur location in human nail. J Invest Dermatol 273–275, 1976
134. Forslind B, Grundin TG, Lindberg M, Roomans G, Werner Y: Recent advances in x-ray microanalysis in dermatology. Scanning Electron Microsc 1985:687–695, 1985
135. Forslind B, Li HK, Malmqvist KG, Wiegleb D: Elemental content of anagen hairs in a normal Caucasian population: studies with proton induced x-ray emission (PIXE). Scanning Electron Microsc 1986:237–241, 1986
136. Craig JM, Goldsmith LA, Baden HO: An abnormality of keratin in harlequin fetus. Pediatrics 46:437–440, 1970
137. Wilkes GL, Nguyen A-L, Wildnauer R: Structure-property relations of human and neonatal rat stratum corneum. I. Thermal stability of the crystalline lipid structure as studied by x-ray diffraction and differential thermal analysis. Biochim Biophys Acta 304:267–275, 1973
138. Elias PM, Bonar L, Grayson S, Baden HP: X-ray diffraction analysis of stratum corneum membrane couplets. J Invest Dermatol 80:213–214, 1983
139. Haftek M, Viac J, Cordier G, Thivolet J: Flow cytometry for separation of keratinocyte subpopulations from viable epidermis. J Invest Dermatol 87:480–484, 1986
140. Pinkus H: Estimation of the epidermis by the strip method of removing horny layers. I. Observations on the thickness of the horny layer, and on mitotic activity after stripping. J Invest Dermatol 16:383–386, 1951
141. Goldschmidt H: Surface area measurements of psoriatic corneocytes: effects of intralesional steroid therapy. J Invest Dermatol 73:558–560, 1979
142. Farrant JL, Rees ALG: Structure of fibrous keratin. Nature 159:535–536, 1947
143. Fukuyama K, Murozuka T, Caldwell R, Epstein WL: Divalent cation stimulation of *in vitro* fibre assembly from epidermal keratin protein. J Cell Sci 33:255–263, 1978
144. King CS, Barton SP, Nicholls S, Marks R: The change in properties of the stratum corneum as a function of depth. Br J Dermatol 100:165–172,1979
145. Christophers E, Kligman AM: Visualization of the cell layers of the stratum corneum. J Invest Dermatol 42:407–408, 1964
146. Mackenzie IC: Ordered structure of the stratum corneum of mammalian skin. Nature 222:881–882, 1969
147. Blair C: Morphology and thickness of the human stratum corneum. Br J Dermatol 80:430–436, 1968
148. Caputo R, Innocenti M, Gasparini G, Peluchetti D: Plasma membranes in psoriatic cells. A freeze-fracture study. J Invest Dermatol 71:245–249, 1978
149. Kitajima Y, Sekiya T, Moir S, Nozawa Y, Yaoita H: Freeze-fracture cytochemical study of membrane systems in human epidermis using filipin as a probe of cholesterol. J Invest Dermatol 84:149–153, 1985
150. Hanau D, Fabre M, Stampf J-L, Grosshans E, Benezra C: ATPase Langerhans cell staining: a technique allowing progression light to electron microscope observation. J Invest Dermatol 86:5–8, 1986
151. Mukai H, Kanzaki T, Nishiyama S: Sulfhydril and disulfide in amyloids of skin-limited and systemic amyloidoses. J Invest Dermatol 82:4–8, 1984
152. Steiner K: A histochemical study of epidermal glycogen in skin diseases. J Invest Dermatol 24:599–618, 1955
153. Lavker RM, Sun T-T: Heterogeneity in epidermal basal keratinocytes: Morphologic and functional correlations. Science 215: 1239–1241, 1982
154. Loomans ME, Hannon DP: An electron microscopic study of the effects of subtilisin and detergents on human stratum corneum. J Invest Dermatol 55:101–114, 1970
155. Scott GH: The localization of mineral salts in cells of some mammalian tissues by micro-incineration. Am J Anat 53:243–279, 1933
156. Cowdry EV, Andrew W: Some cytochemical and cytologic features of senile keratosis. J Gerontol 5:97–111, 1950
157. Wei X, Roomans GM, Forslind B: Elemental distribution in guinea-pig skin as revealed by x-ray microanalysis in the scanning transmission microscope. J Invest Dermatol 79:167–169, 1982
158. Warner RR, Myers MC, Taylor DA: Electron probe analysis of human skin: Element concentration profiles. J Invest Dermatol 90:78–85, 1988
159. Grundin TG, Roomans GM, Forslind B, Lindberg M, Werner Y: X-ray microanalysis of psoriatic skin. J Invest Dermatol 85:378–380, 1985
160. Holbrook KA, HolbrookJR, Odland GF: A comparison of the effects of sample preparation on the X-ray microanalysis and ultrastructure of thin sectioned specimens of human skin. Scanning Electron Micros 1976,1, 265–274, 1976
161. Warner RR, Myers MC, Taylor DA: Electron probe analysis of human skin: determination of the water concentration profile. J Invest Dermatol 90:218–224, 1988
162. Kurz K, Steigleder GK, Bischof W, Gonsior B: PIXE analysis in different stages of psoriatic skin. J Invest Dermatol 88:223–226, 1987
163. Moberger G, Engstrom A: Historadiographic studies on normal, hyperplastic and cancerous epidermis. J Invest Dermatol 22:477–491,1954
164. Tidman MJ, Eady RAJ: Evaluation of anchoring fibrils and other components of the dermal-epidermal junction in dystrophic epidermolysis bullosa by a quantitative ultrastructural technique. J Invest Dermatol 84:374–377, 1985
165. Van Neste DJJ, Staquet M-J, Leroy BP, De Coster W: Distribution pattern of psoriatic keratoblasts: computer-assisted image-analysis for combined evaluation of DNA synthesis and expression of 67 kD keratin polypeptides in the epidermis of stable plaques of psoriasis. J Invest Dermatol 90:382–386, 1988
166. Rowden G: Ultrastructural studies of keratinizing epithelia of the mouse. III. Determination of the volumes of nuclei and cytoplasm of cells in murine epidermis. J Invest Dermatol 64:1–3,1975
167. Bergstresser P, Chapman SL: Maturation of normal human epidermis without an ordered structure. Br J Dermatol 102:641–648,1980
168. Tammi R, Jansen CT, Santti R: Histometric analysis of human skin in organ culture. J Invest Dermatol 73:138–140, 1979
169. Watt F, Green H: Involucrin synthesis is correlated with cell size in human epidermal cultures. J Cell Biol 90:738–742, 1981
170. Klein-Szanto AJP: Stereologic baseline data of normal human epidermis. J Invest Dermatol 68:73–78, 1977
171. Sagebiel RW, Reed TH: Serial reconstruction of the characteristic granule of the Langerhan's cell. J Cell Biol 36:595–602,1968
172. Braverman IM, Keh-Yen A: Ultrastructure and three-dimensional reconstruction of several macular and papular telangiectases.J Invest Dermatol 81:489–497, 1983
173. Braverman IM, Keh-Yen A: Ultrastructure of the human dermal microcirculation: the horizontal plexus of the papillary dermis. J Invest Dermatol 66:131–142, 1976